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Title: DNA Precursor Compartmentation in Mammalian Cells: Metabolic and Antimetabolic Studies of Nuclear and Mitochondrial DNA Synthesis.

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This dissertation describes an investigation of DNA precursor supply for mitochondrial and nuclear DNA synthesis in HeLa cells and Mouse L-cells. HeLa cells were used for the quantitation of cellular and mitochondrial deoxyribonucleoside triphosphate (dNTP) and ribonucleoside triphosphate (rNTP) pools and of changes in pools in response to treatment with the antimetabolites methotrexate (mtx) and 5-fluorodeoxyuridine (FUdR). Use of an enzymatic assay for dNTPs and of improved nucleotide extraction methods allowed quantitation of mitochondrial dNTP pools. All four mitochondrial dNTP pools expand following treatment with mtx or FUdR whereas cellular dTTP and dGTP pools are depleted. Mitochondrial rNTP pools were also found to expand in response to these antimetabolites. The cellular dGTP pool is severely depleted by mtx and FUdR' which indicates dGTP levels might play an important role in mtx- and FUdR-induced cytotoxicity.

Mouse L-cells were used to determine the relative contributions of an exogenously supplied precursor to nuclear and mitochondrial DNA replication. These experiments provide a dynamic representation of mitochondrial and nuclear DNA precusor metabolism. Cells were labeled to near steady state specific activities with ^{32}P-orthophosphate and subsequently labeled with [^{3}H]uridine, a general pyrimidine precursor, in the continuing presence of ^{32}P. Cells were harvested at time intervals up to four days after [^{3}H]uridine addition and mitochondrial and nuclear DNAs were purified. Deoxyribonucleoside monophosphates derived from these DNAs were separated by HPLC and the $^{3}H/^{32}P$ ratio in each pyrimidine determined. The dCMP residues in mitochondrial DNA (mtDNA) were found to be derived exclusively from the exogenous supplied uridine. The dTMP residues from nuclear and mtDNA and the dCMP residues from nuclear DNA were seen to be synthesized partly from exogenous sources and partly from other sources, presumably de novo pyrimidine synthesis. The results suggest mtDNA replication can utilize exogenous dCTP precursors more efficiently than nuclear replication.

Combined these results demonstrate that nuclear and mitochondrial DNA replication are supplied by distinct dNTP pools and regulation of dNTP biosynthesis is by different mechanisms in each of these two compartments.

DNA Precusor Compartmentation in Mammalian Cells: Metabolic and Antimetabolic Studies of Nuclear and Mitochondrial DNA Synthesis

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

DNA Precursor Compartmentation in Mammalian Cells: Metabolic and Antimetabolic Studies of Nuclear and Mitochondrial DNA Synthesis

I. INTRODUCTION

Nearly a quarter of a century has elapsed since Kornberg and his colleagues demonstrated the presence of enzymes capable of carrying out DNA dependent DNA synthesis in Escherichia coli (1). Since then the details of DNA replication have been partially elucidated by work on bacteria, in particular Escherichia coli, their phages, and vertebrate cells, particularly mammalian cells. The subcellular construction of eukaryotic cells combined with the enormous amount of DNA present in each cell would a priori lead one to expect a much more complicated replication mechanism than is found in bacteria. Indeed this is the case and is evident by the following: 1) compartmentation of the cell's DNA and DNA replication apparatus in the nucleus and mitochondria which are separated by semi-permeable membranes from the cytoplasm, 2) the temporal control of DNA replication such that replication occurs during a six to eight hour period, the S-phase, representing one-third to one-fourth of the total cell cycle, and 3) the organization of nuclear DNA as chromatin forming a highly compacted protein-DNA complex that may affect the rate of replication in eukaryotic cells.

The proximal precursors of DNA synthesis are the deoxyribonucleoside triphosphates (dNTPs) which represent highly specialized molecules within the cell. Other than their involvement in glycoprotein biosynthesis as nucleotide sugars, dNTPs function

TABLE I. ABBREVIATIONS USED

Abbreviation	Meaning
BSA	bovine serum albumin
CH_2 THF	methylene tetrahydrofolate
CH THF	methenyl tetrahydrofolate
CHO THF	formyl tetrahydrofolate
DEAE	diethylaminoethyl
DHF	dihydrofolate
DNAse	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraacetic acid
EtBr	ethidium bromide
FdUMP	5-fluorodeoxyuridylate
FUdR	5'-fluorodeoxyuridine
GFC	glass fiber filter
HPLC	high performance liquid chromatography
IMP	inosinate
kb	kilobases
MOPS	morpholinopropane sulfonic acid
mtDNA	mitochondrial DNA
NaPPi	sodium pyrophosphate
OMP	orotidylate
PPi	pyrophosphate
PRPP	5-phosphoribosyl 1-pyrophosphate
RNAse	ribonuclease
rNTP	ribonucleoside triphosphate
SDS	sodium lauryl sulphate
SMED	250 mM sucrose, 20 mM MOPS, pH 7.0, 3 mM EDTA, 2 mg/ml digitonin
SP-sephadex	sulfopropyl sephadex
SSC	150 mM NaCl, 15 mM sodium citrate
TCA	trichloroacetic acid
TdR	deoxythymidine
TEAB	triethylammonium bicarbonate
Tris	(Tris-hydroxymethyl) aminoethane
TSE	10 mM Tris-HCl, 10 mM NaCl, 0.25 mM EDTA, pH 7.6
UdR	deoxyuridine
UR	uridine
XMP	xanthylate

solely as DNA precursors. Conversely, ribonucleoside triphosphates (rNTPs) play several major roles in cellular metabolism other than utilization as RNA precursors. Furthermore, the requirement for dNTPs during DNA replication occurs during a small portion of the mammalian cell cycle and is limited to a relatively small number of sites when compared to other macromolecular biosyntheses. Consideration of these characteristics leads one to expect a very tight control over the level of dNTP synthesis and the location at which dNTPs are formed. Indeed articles by Garrett and Santi (2) indicate dNTP pools are 100-fold lower than those of the corresponding rNTPs.

Because of these considerations DNA precursor biosynthesis has been the target of numerous antiproliferative drugs designed to combat neoplasia, as well as viral and bacterial infections. These antimetabolites interfere with DNA precursor biosynthesis resulting in a specific inhibition of DNA synthesis without affecting other areas of metabolism. The enzymes responsible for thymine nucleotide biosynthesis have for many years been recognized as suitable targets for two classes of antimetabolites, the fluorinated pyrimidines and the folate antagonists.

This dissertation is concerned with the regulation of DNA precursor biosynthesis in mammalian cells, particularly mammalian mitochondria, and how antimetabolites, such as the fluorinated pyrimidines and the folate antagonists, disrupt the metabolism of DNA precursors. Separate sites of DNA precursor biosynthesis within the cell are seen as important determinants in the overall regulation of DNA precursor biosynthesis in normal cells and those subjected to stress by antiproliferative drugs.

A. Deoxyribonucleotide Biosynthesis in Mammalian Cells

As mentioned above, the immediate precursors for DNA biosynthesis are the 2'-deoxyribonucleoside 5'-triphosphates (dNTPs), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP) and deoxyadenosine triphosphate (dATP). Rarely occurring more often under certain metabolic stresses (3,4), deoxyuridine triphosphate (dUTP) is utilized in place of dTTP. The consequences and cellular mechanisms for dealing with such an event will be discussed in detail in a later section. The evidence that dNTPs are the proximal precursors for DNA synthesis is extensive. All known DNA polymerases utilize 2'-deoxyribonucleoside 5'-triphosphates most efficiently and exclusively in comparison to 2'-deoxyribonucleosides at a lower level of 5' phosphorylation. No DNA polymerase is known to utilize 2'-deoxyribonucleoside 3'-triphosphates and biosynthetic pathways for their production have not been found. Finally, all DNA polymerases have an absolute requirement for a free 3'-hydroxyl on the deoxyribose of the primer terminus and require a 5'-triphosphate for chain elongation (5,6).

The primary source of dNTPs is de novo synthesis of the nucleotides. As shown in Figure 1 dNTPs are formed de novo without passage through intermediate steps involving the free bases or nucleosides. Alternative routes to dNTPs are the salvage pathways used to recycle the free bases and nucleosides produced by the breakdown of nucleic acids and to utilize exogenous sources of free bases and nucleosides.

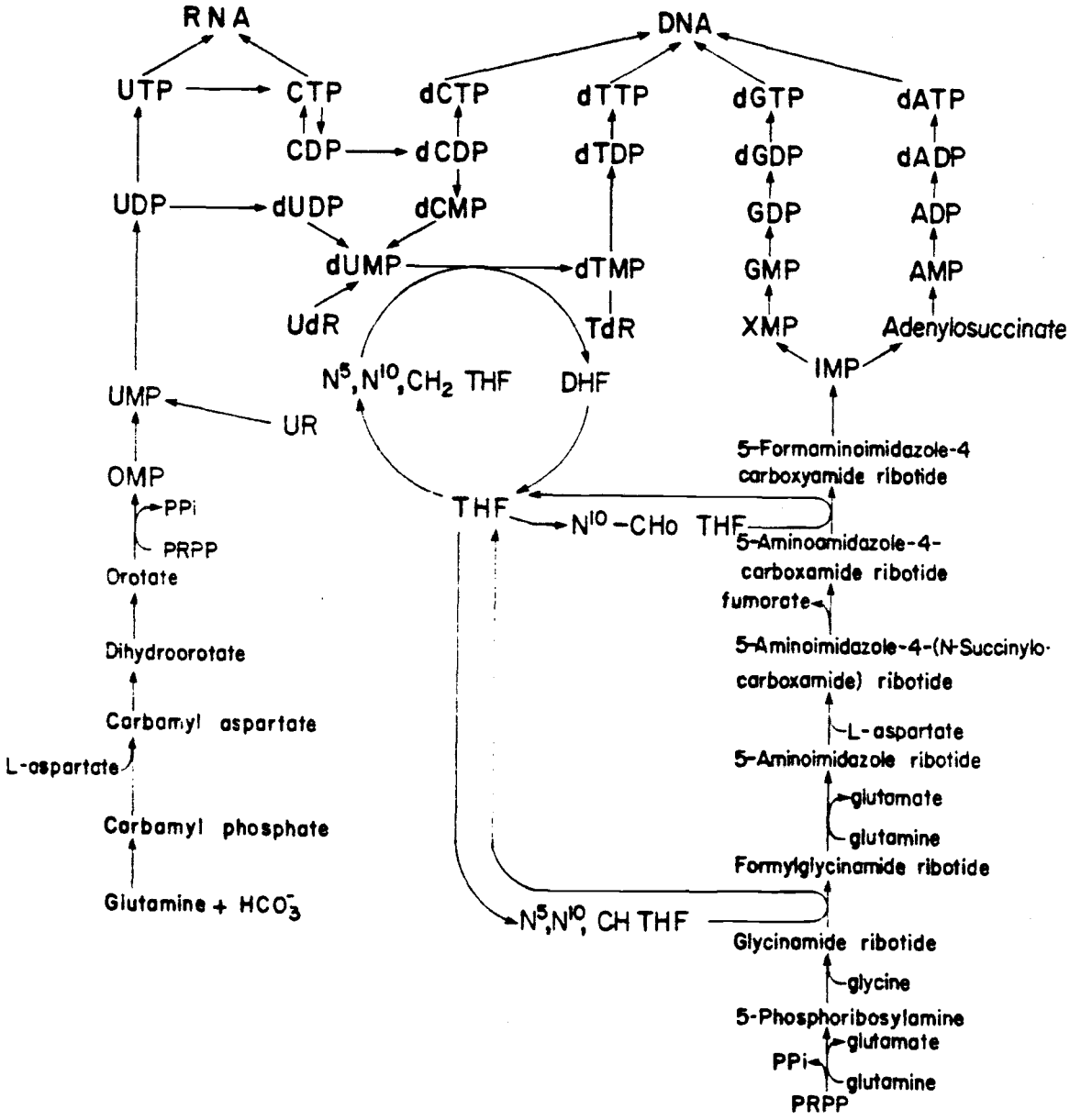

Figure. 1. Pathways of DNA precursor biosynthesis in mammalian cells. Abbreviations are listed in Table I. Entrance of UR, UdR, and TdR into the biosynthetic pathways are *via* salvage enzymes; the remainder represent *de novo* biosynthesis

B. De Novo Pyrimidine Biosynthesis

In mammalian cells, as in prokaryotes, six enzymatic reactions are required for de novo biosynthesis of UMP (Figure 1). Contrary to prokaryotes, the enzyme functions in mammalian cells are located on only three proteins (49). Located on one mutifunctional protein are the first three enzyme activities: carbamyl phosphate synthetase, aspartate transcarbamylase, and dihydroorotase. Carbamyl phosphate synthetase is the major regulatory step in mammalian pyrimidine biosynthesis. It is feedback inhibited by several pyrimidine nucleotides and activated by 5-phosphoribosyl 1-pyrophosphate (PRPP). The next enzymatic reaction, dihydroorotate dehydrogenase, oxidizes dihydroorotate to orotate, is associated with a monofunctional protein, and is located on the outer surface of the inner mitochondrial membrane. Orotate phosphoribosyltransferase condenses orotate with PRPP to yield orotidylate, which is subsequently converted to uridylate (UMP) by orotidylate decarboxylase. These last two enzymatic activities are also located on one multifunctional protein. Nucleoside monophosphokinase converts UMP to UDP, which is the branch point for commitment to either uridine triphosphate (UTP) and cytidine triphosphate (CTP) or to dTTP. If UDP is phosphorylated to UTP by nucleoside diphosphokinase it can then be subsequently used for RNA synthesis or conversion to CTP by CTP synthetase. CTP is also used for RNA synthesis but can be used as a precursor for the synthesis of dTTP as described below.

Cellular dTTP is produced by any one of four distinct pathways (Fig. 1). The key metabolic intermediate is deoxyuridylate which is involved in three of the four pathways. Deoxyuridylate

(dUMP) can be produced through salvage of deoxyuridine by thymidine kinase, by reduction of UDP to dUDP and conversion to dUMP, and by the deoxycytidylate deaminase reaction. Thymidine can be salvaged by thymidine kinase and is the fourth route to dTTP. Methylation of dUMP by thymidylate synthetase occurs by transfer of the methylene group from N^5,N^{10}-methylenetetrahydrofolate accompanied by a reduction of the group, at the expense of tetrahydrofolate, to form deoxythymidylate (dTMP). Thus deoxythymidine nucleotides are unique in comparison to the other four deoxynucleotides in that deoxythymidylate is formed at the monophosphate level whereas the others are formed at the diphosphate level from the corresponding ribonucleotides by the action of ribonucleotide reductase. Thymidylate synthetase is a pivotal enzyme in the overall biosynthesis of DNA because it represents the only de novo source of deoxythymidine nucleotides.

C. De Novo Purine Biosynthesis

Biosynthesis of the purine ring differs from de novo pyrimidine synthesis in that the primary product inosinate (inosine 5'-phosphate, IMP) is formed by sequential addition of NH_3, glycine, CHO, NH_3, CO_2, aspartate and CHO groups to PRPP as shown in Figure 1. The purine ring is constructed at the nucleotide level to yield IMP, whereas de novo pyrimidine synthesis forms a free pyrimidine ring, orotate, which is subsequently attached to PRPP.

Inosinate represents a branch point in de novo purine nucleotide synthesis much as uridylate does in pyrimidine biosynthesis. Adenylate (AMP) is formed from inosinate by two enzymes, adenylosuccinate synthetase and adenylosuccinase. Guanylate synthesis (GMP) is also a two step process, formation of xanthylate

(XMP) by inosinate dehydrogenase, followed by xanthylate aminase yielding guanylate.

D. Salvage Pathways

Cells have the ability to utilize preformed nucleosides and free bases that arise from either extracellular fluids or breakdown of nucleic acids. These processes supplement the de novo synthesis of nucleic acid precursors and become vital to the cell when mutations, drugs, or disease block de novo pathways. Salvage enzymatic activities include phosphoribosyl transferases and nucleoside phosphorylase for conversion of free bases, nucleoside kinase for utilization of nucleosides and deaminases responsible for base alterations of nucleosides and nucleotides. An example of the latter group as well an indication of the diversity of salvage paths in animal cells is adenosine deaminase. Humans lacking this enzyme suffer from severe combined immunodeficiency disease. This genetic disorder affects the immune system probably because intracellular dATP accumulates in cells of the immune system blocking DNA synthesis by inhibition of ribonucleotide reductase (46,47). Why other cells, even rapidly dividing cells, are unaffected may be due to the relatively higher level of nucleoside kinase in lymphoid tissues (127).

E. Ribonucleotide Reductase

The reduction of ribonucleotides to deoxyribonucleotides occurs at the diphosphate level by the enzyme system known as ribonucleotide reductase. This enzyme may ultimately prove to be the rate-limiting step for production of DNA precursors. The allosteric regulation of E. coli ribonucleotide reductase is complex but well understood. The mammalian enzyme is equally complex and so far has

demonstrated a similarity to the bacterial enzyme (7). As shown in Figure 2, there is a cascade of allosteric activations initiated by ATP followed by inhibition of the entire complex by dTTP and dATP. This allows the cell to fine tune the reduction of ribonucleotides to actually meet the needs of DNA replication.

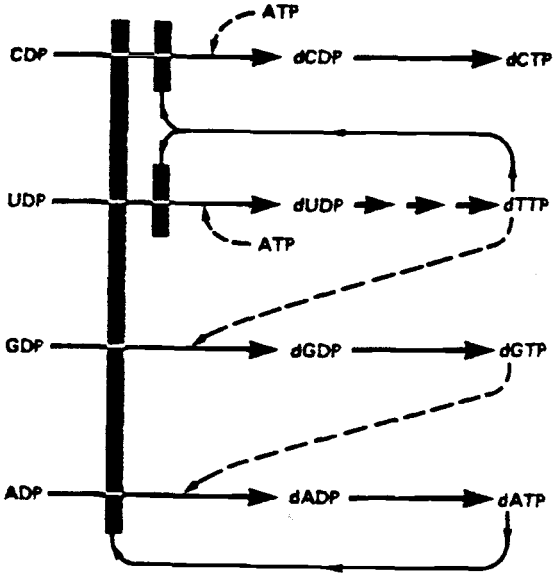

Figure 2. Regulation of ribonucleotide reductase from E. coli. Solid lines indicate allosteric inhibitions and broken lines activation. From Kornberg (6).

F. Enzymes of DNA Replication

Animal cells possess at least three distinct DNA polymerases; α, β, and γ. Both α and β are found in the nucleus (8,9,10,11) whereas γ is found in the mitochondria as well as the nucleus (12,13). The most active is polymerase α, which is also closely associated with chromosomal replication. In growing cells polymerase α is responsible for at least 85% of DNA polymerase activity whereas in quiescent cells it accounts for only about 5% (6). The *in vitro* measured DNA synthetic rate of α, 30 nucleotides per second (14), approaches that of the *in vivo* rate of fork movement, 50-100 nucleotides per second (15,16). The number of polymerase α molecules per cell combined with the synthetic rate can easily account for replication of cellular DNA during the S-phase (6). Polymerase β is thought to play a role in repair and recombination. Cells stressed by agents which damage DNA show increased levels of β activity (17). The β activity in quiescent cells, where only DNA repair synthesis is occurring, is high relative to the other polymerases. The synthetic activity of polymerase β is ten times lower than α and when combined with the number of molecules of β per cell cannot account for chromosomal replication (6). Both α and β polymerases have numerous accessory proteins. What role these play *in vivo* and whether the *in vitro* measurements are artifactual because one or more accessory proteins are missing remains unclear.

Polymerase γ is the mitochondrial enzyme responsible for replication of mitochondrial DNA (12). It possesses many novel biochemical characteristics which may prove to be linked to the rather

unusual mechanism of mitochondrial DNA (mtDNA) replication discussed below.

G. Mitochondrial DNA Structure

The mitochondrial genome of all metazoan animals is a small circular molecule that is remarkably constant in both size and function. The mtDNAs from a variety of sources, including mammalian, vertebrate, invertebrate, and avian cells all fall within a narrow size range of about 15 to 17 kb of DNA. Mitochondria are known to contain DNA, RNA, and protein synthesizing capabilities that are physically segregated from the rest of the cell. Although some of these synthetic functions are in part comprised of mtDNA gene products most are the result of nuclear genes whose products are imported into the mitochondria. Recently publication of the 16,569-base pair human (21) and the 16,295-base pair mouse (22) mtDNA sequences have provided a vast amount of information concerning mitochondrial biogenesis and mitochondrial molecular biology (23,24).

The mammalian mtDNA sequence information that is available indicates the gene organization and mode of expression is identical for human, mouse, bovine, and rat mtDNAs (24). It seems likely that this observation will be extended to at least all vertebrates within the animal kingdom. Encoded by mtDNA are the genes for two ribosomal RNAs, 22 transfer RNAs, and 13 proteins. These genes are transcribed and the mRNAs translated within the organelle. Of the 13 mitochondrial translation products five have been assigned a function. These comprise in part the enzyme complexes of the inner mitochondrial membrane. The identified genes are for the three largest subunits of cytochrome oxidase, a subunit of the ATPase

complex, and cytochrome c of the cytochrome bc_1 complex. The eight remaining mitochondrial encoded proteins have not been identified. Evidence that they exist comes in part from the observation that their intact reading frames are conserved in human, bovine, and mouse mtDNAs and polyadenylated mRNAs which map in the corresponding genes have been identified (22). They are classic examples of genes in search of functions.

Mammalian mitochondrial molecular biology derived from DNA sequence information has proven to emphasize an unprecedented economy of genetic information. There are no introns in any genes, very few if any non-coding bases between genes, the mRNAs lack both 3' and 5' non-translated leader and trailing regions, and some mRNAs generate stop codons only after polyadenylation of the 3' termini. The translational apparatus utilizes only 22 tRNAs, rRNAs of reduced size compared to all other known rRNAs, and a unique genetic code that is the only exception to the universal character of the genetic code found elsewhere in biology (21,22,23,24).

H. Mitochondrial DNA Replication

The general mechanism of mtDNA replication is known in greatest detail in mouse L-cells and is termed asynchronous displacement replication (18,19,20). Replication of animal mtDNA has recently been reviewed by Clayton (45). Several physical properties have facilitated studies of mouse L-cell mtDNA replication and mammalian mtDNA replication in general. The mtDNA can be separated into heavy (H) and light (L) strands by equilibrium centrifugation in alkaline CsCl and most of the replicating intermediates can be isolated as covalently closed circular DNA. Although the mouse L-cell

system is commonly considered as representative of mammalian mtDNA replication, some differences have been noted in other animal cell systems.

The replication of mouse L-cell mtDNA begins with the displacement synthesis of a specific 550 to 670 nucleotide segment (7S mtDNA) of the H-strand (25). The structure that is formed is called the D-loop and mtDNA with this structure is termed D-mtDNA (20,26). Up to 70% of the mtDNA molecules contain this D-loop (27). Replication continues by the selective extension of a 7S mtDNA molecule until the L-strand origin of replication has been passed and the daughter H-strand is approximately 60% replicated. Not all 7S mtDNA molecules serve as H-strand replication primers because they are being constantly degraded and resynthesized with a mean half-life of 90 minutes (26,28). Once the L-strand origin of replication has been exposed as a single-stranded region, initiation occurs at a precise location on the displaced parental H-strand (22,29) and L-strand synthesis proceeds in the direction opposite to H-strand synthesis. Once both strands have been completely replicated the resultant daughter molecules are slowly converted from closed circular molecules lacking superhelical turns to supercoiled forms with approximately 100 superhelical turns (30).

Some variations in this general mechanism are seen in several mammalian species. The D-loops of mouse (28), rat (31), sheep and goat (32), and hamster (33) are all similar in size but the relative abundance of D-mtDNA varies greatly from a very low frequency in HeLa cells (35) to the 70% frequency in mouse L-cells. The degree

of asymmetry of replication of the two complementary strands of mtDNA also varies significantly with different species (33).

The time course of a complete round of mammalian mtDNA replication has been determined to be 60 minutes for mouse L-cells (26,28) and 120 minutes for hamster cells (34) by using pulse chase labeling experiments. There appears to be no cell cycle dependence on mtDNA replication for mouse L-cells (36) or yeast (37). Molecules are constantly being selected for replication at random throughout the cell-cycle and once initiated proceed at an average synthetic rate of less than five nucleotides per second. This is an order of magnitude slower than the polymerization rate of nuclear replicons (15,16) and could be due to either topological constraints or limiting concentrations of dNTPs. The former is considered more likely since the rate of L-strand synthesis is considerably faster than that of the H-strand in mouse L-cells (38). It is thought that L-strand synthesis proceeds rapidly because the H-strand template is single-stranded and there is no need to unwind the parental duplex. This would imply the mouse L-cell mitochondrion has the enzymatic machinery and nucleotide supply necessary to rapidly synthesize DNA but topological constraints hinder H-strand synthesis, therefore lowering the overall average synthetic rate.

I. Mitochondrial Deoxynucleotide Metabolism

Very little is known about mitochondrial metabolism of deoxynucleotides or the enzymes responsible for their metabolism. What is known suggests the existence of a anabolic apparatus capable of autonomous synthesis of deoxynucleotides. Several deoxynucleotide metabolizing enzymes including mouse and human thymidine kinase

(40,42), human deoxycytidine kinase (44), calf thymus deoxyguanosine kinase (41), and rat deoxythymidine 5'-nucleotidase (43) have been shown to exist in unique mitochondrial forms.

J. Folate Antagonists and Fluorinated Pyrimidines as Antimetabolites

For years it has been known that the folate antagonist methotrexate and 5-fluoro-deoxyuridylate (FdUMP) are stoichiometric inhibitors of their respective target enzymes (50,51). Methotrexate indirectly inhibits the de novo synthesis of thymidylate by depletion of the one-carbon group donor, 5,10-methylene tetrahydrofolate, the required carbon donor in the thymidylate synthetase reaction which methylates dUMP to thymidylate (Fig. 1). The methotrexate-induced depletion of 5,10-methylene tetrahydrofolate is caused by an inhibition of dihydrofolate reductase, the enzyme responsible for regeneration of tetrahydrofolate from dihydrofolate (55,56). FdUMP directly inhibits thymidylate synthetase by forming a covalent complex with the enzyme in the presence of 5, 10-methylene tetrahydrofolate (52,53,54). The voluminous literature on these drugs has been reviewed (54,56) and only the primary references are cited here.

A blockade of thymidylate synthetase in the presence of these inhibitors would obviously exert drastic effects on tissues that require a high rate of DNA synthesis. Because neoplastic cells fall into this category, thymidylate synthetase has for years been the target for the design of antiproliferative chemotherapuetic agents such as FdUMP and methotrexate. The cell has only one route to circumvent an inhibition of thymidylate synthetase; thymidine kinase, a salvage enzyme which converts thymidine to thymidylate (Fig. 1). Tissues generally do not have available an adequate supply of

thymidine to overcome the effects of these antimetabolites and eventually the net result is cell death. A serious drawback to using drugs that inhibit DNA precursor biosynthesis is the considerable toxicity to normal rapidly proliferating cells such as gastrointestinal cells and cells of the hematopoietic tissues. The exact mechanism of cell death by inhibition of DNA synthesis has not as yet been determined despite nearly 30 years of intense effort on the part of many investigators. In general the phenomenon caused by antimetabolites or conditions which deplete the thymidylate pool has been referred to as "thymineless death" (56,58).

As a consequence of thymidylate synthetase inhibition, as caused directly by FdUMP or indirectly by methotrexate, the cellular dTTP concentration is reduced (77,78,79) and the dUMP pool is increased dramatically (59,60,61,74). The dTTP drop is due to its utilization in DNA synthesis without replenishment. Adding to the increase in dUMP is the "de-inhibition" of dCMP deaminase because of the fall in the level of dTTP which has a strong inhibitory effect on dCMP deaminase (61,62). Secondary contributions to the dUMP pool are increased activities of UDP reductase and CDP reductase in the presence of lower dTTP levels and thymidine kinase which salvages deoxyuridine to dUMP (61,73).

As a result of these observations the dUTP/dTTP ratio in thymidylate depleted cells might be expected to increase significantly. Strongly supporting this are the observations of Goulian and co-workers, who have shown, using methotrexate treated human lymphoblasts, an increase of at least 10^4 in the dUTP/dTTP ratio (3,63). The enzyme deoxyuridine triphosphate nucleotidohydrolase

(dUTPase) which hydrolyzes dUTP to dUMP and PPi normally prevents the accumulation of dUTP in both bacterial (64,65) and mammalian (66,67) cells. Apparently the enzyme's capability is greatly exceeded by the large increase in substrate (dUTP) concentration.

If dUTP is present it is incorporated into DNA in animal cells (4) but is normally removed by the enzyme uracil-DNA glycosylase, which removes the uracil base leaving an apyrimidinic site subject to normal cellular excision-repair processes. Resynthesis of DNA in the repaired area would cause incorporation of additional dUMP and reinitiate the repair process. Consequently, these repair processes would probably lead to extensive degradation and probably unrepairable, lethal, double-stranded breaks in the DNA (3). Therefore increased dUTP/dTTP ratios allow an increased frequency of that normally very rare event, the incorporation of dUMP into DNA. Two groups have shown this to be a consequence of antifolate treatment on human lymphoblasts (3,69). The deleterious effects of methotrexate-induced misincorporation of uracil into DNA have not as yet been proven but are consistent with several observations that link thymidine deprivation with damage to DNA: 1) inhibition of thymidylate synthetase results in DNA fragmentation in animal cells (68) and 2) prokaryotic cells that are deprived of thymine or the mechanisms necessary to prevent dUMP incorporation into DNA exhibit damage to DNA and growth inhibition (70,71,72). Thus, the cytotoxic effect of methotrexate or FdUMP could be due to the inclusion of dUMP in DNA followed by extensive degradation of the DNA by cellular repair processes.

An important consideration regarding methotrexate cytotoxicity has been advanced by Moran, Mulkins, and Heidelberger (73). They proposed that the activity of thymidylate synthetase has an important role in the development of methotrexate cytotoxicity. Because thymidylate synthesis causes oxidation of tetrahydrofolate to dihydrofolate (Fig. 1), the rate of this process in the presence of methotrexate would have an important effect on the rate of tetrahydrofolate depletion. The other major cellular processes which require folate coenzymes are the purine nucleotide biosynthetic pathway (Fig. 1) and biosynthesis of glycine and methionine. Thymidylate biosynthesis is novel in this regard because it is the only reaction which oxidizes tetrahydrofolate to the dihydro level. Therefore, in cells that are supplied exogenous tetrahydrofolates the only role of dihydrofolate reductase is to regnerate tetrahydrofolate from the dihydrofolate generated in the thymidylate synthetase reaction. When dihydrofolate reductase is inhibited by methotrexate the depletion of tetrahydrofolate is directly related to the activity of thymidylate synthetase. There is experimental evidence that control of thymidylate synthetase in eukaryotic cells is not by dTMP feedback inhibition but rather by the availability of dUMP (61). Methotrexate treated cells with elevated dUMP pools have a rapid depletion of tetrahydrofolates due to flux through thymidylate synthetase and as a consequence the other bio-synthetic pathways which require reduced folates would be inhibited. Others have reported evidence that dTMP feedback inhibition of thymidylate synthesis occurs in L1210 mouse leukemia cells (48). In this case the rate of methotrexate-induced depletion of reduced folates would decrease. Although these

observations of Moran et al. (73) go a long way toward interpretation of previously poorly understood pharmacological data, there is no conclusive evidence that reduced levels of tetrahydro-folates are directly responsible for methotrexate cytotoxicity.

Another possible component of methotrexate cytotoxicity is the depletion of purines as a consequence of tetrahydrofolate depletion. Several reports have indicated disturbances of de novo purine biosynthesis in cells exposed to methotrexate (75,76,80). The effects of methotrexate on RNA synthesis (indicative of purine metabolism) are not related to cytotoxicity (76). Currently available data can at best attribute purine deprivation in conjunction with thymidylate depletion as important in causing cytotoxicity.

Despite the numerous studies that have examined the biochemical effects of methotrexate in cultured cell lines in vivo, a consistent and precise mechanism has not appeared. The most apparent feature of these studies is the dramatic differences observed on a variety of biochemical parameters when different cell lines are investigated. Because this thesis focuses on dNTP metabolism in HeLa and mouse L-cells a discussion of these differences with respect to dNTPs is appropriate.

A consistent observation has been the methotrexate-induced reduction in cellular dTTP levels. Human leukemic CCRF-CEM cells in the presence of 5×10^{-7} M methotrexate showed immediate decreases in dCTP and dTTP but a somewhat slower reduction in dATP and dGTP levels (81). Human oesteosarcoma cells in the presence of 1×10^{-7} M methotrexate exhibited lowered dTTP and dGTP levels (82). Using mouse lymphoma L5178Y cells it was shown 1×10^{-6} M methotrexate causes

increased levels of dCTP, unchanged levels of dATP and, 50% lower levels of dGTP and dTTP (83). When five different mammalian cell lines were investigated for dNTP sensitivity to methotrexate, it was found that dTTP pools decreased while purine dNTP levels were unchanged or increased (84). Mouse leukemia L1210 cells in the presence of 2.5×10^{-8} M methotrexate showed decreases in all four dNTP pools with dTTP being much more sensitive to methotrexate (79). Undoubtedly some of these results vary to a degree as a result of experimental design, methotrexate concentration, and analytical methods (85). But just as obvious are the clearly different perturbations in dNTP pools in different cell lines under identical conditions of pharmacological insult.

K. Antimetabolites and Mitochondrial DNA Synthesis

This thesis is primarily concerned with the role mitochondria have in the cellular biosynthesis of deoxyribonucleotides. As an aid in probing this role the antimetabolites methotrexate and fluorodeoxyuridine were used to perturb cellular DNA precursor biosynthesis. Differential effects on mitochondrial and nuclear DNA synthesis were observed by Bogenhagen and Clayton (91) in cultured mouse L-cells following administration of either methotrexate or fluorodeoxyuridine. Their observation was that mitochondrial DNA synthesis is relatively resistant to these drugs and they rationalized that mitochondrial DNA synthesis is more resistant to dTTP depletion than is nuclear DNA synthesis. An alternative explanation for their results is that DNA precursor biosynthesis is independently regulated in the nucleus and mitochondrion. Determination of the subcellular localization of dNTPs in the presence and absence of methotrexate or

fluorodeoxyuridine would indicate whether mitochondrial DNA precursor biosynthesis is indeed resistant to these antimetabolites. These experiments required the use of highly sensitive analytical methods for the quantitation of dNTPs as well as development of methods to rapidly isolate and quantitate mitochondria from cultured mammalian cells.

Because intracellular levels of dNTPs are very low and mitochondrial dNTPs expected to be even lower, quantitation requires an assay with picomolar sensitivity. An enzymatic assay with a sensitivity of at least one picomole was developed by Solter and Handschumacher (86) and improved by others (87,88). This enzymatic method assays the level of any one dNTP by determining the incorporation of a radioactively labeled counternucleotide into a synthetic, alternating, copolymeric DNA template in the presence of E. coli DNA polymerase I (Fig. 3). This enzymatic assay has several advantages over two alternative analytical methods, high performance liquid chromatography (HPLC) (2) or equilibrium labeling of cellular dNTPs with ^{32}P (86). HPLC is sensitive only down to 30 pmoles and equilibrium labeling of cells with ^{32}P requires long labeling periods and subjects cells to radioisotopic damage. Because of these considerations the enzymatic assay was used to determine mitochondrial dNTP levels.

This work required a rapid method for the isolation of mitochondria from cultured mammalian cells. A voluminous literature, which has been reviewed (90), exists on mitochondrial isolation methodology. Unfortunately these methods have, in general, been developed for tissues, particularly rat hepatocytes. Very little has

Enzymatic Assay For Deoxyribonucleoside Triphosphates

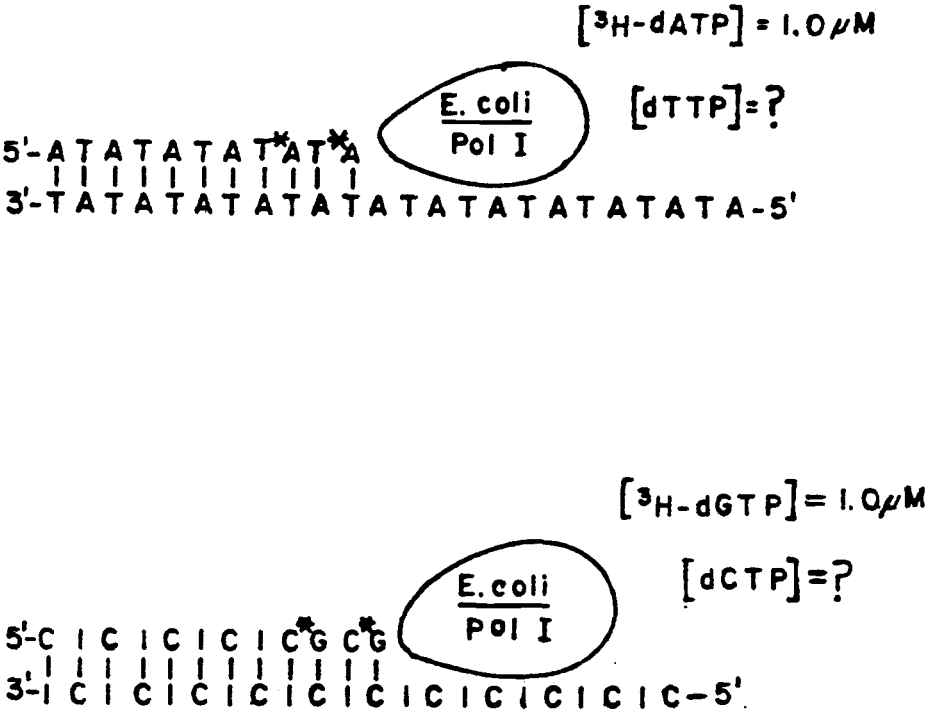

Figure 3. Enzymatic assay for deoxyribonucleoside triphosphates.

been published concerning isolation methods for cultured mammalian cells. The mitochondrial isolation method applied to the work presented here is a combination of a technique utilizing selective membrane solubilization properties of digitonin, a steroid glycoside, and a differential centrifugation method for isolation of HeLa cell and mouse L-cell mitochondria (92). The initial observation by Zuurendonk and Tager (93) that digitonin increases the permeability of rat hepatocytes allowed development of rapid mitochondrial isolation methods. The digitonin-induced membrane solubilization is believed to be the result of the stoichiometric binding of digitonin to cholesterol and other β-hydroxysterols present in the plasma membrane (94). This effect is plasma membrane-specific due to the severalfold higher concentration of cholesterol in the plasma membrane relative to intracellular membranes, expecially mitochondrial membranes (95). Other membrane solubilization agents such as triton X-100, NP-40, or toluene are, at best, much less plasma membrane-specific. Digitonin-induced membrane solubilization has been used to study mitochondrial metabolites (96,97) and proteins (93,98,99). The work described in this thesis extends the utilization of digitonin to include isolation of metabolites (dNTPs) present in minute quantities from large numbers of cultured HeLa cells.

The rationale for using HeLa cells to study the effects of antimetabolites on mitochondrial dNTP pools stems from the observation of Bogenhagen and Clayton (92) that these cells contain about eight times as much mtDNA as mouse L-cells. A larger quantity of mtDNA could require larger dNTP pools for replication and hence HeLa cells might be a superior cell line for measuring dNTP pool perturbations in

these organelles. This discrepancy in mtDNA mass is not due entirely to differences in cell size, organelle number, or in the mitochondrial volume to cell volume ratio since these values are within a factor of two for HeLa and mouse L-cells (92). The reason is probably rooted in the rather loose control of mtDNA copy number, an area which is poorly understood (45).

L. Mitochondrial Deoxypyrimidine Pool Dynamics

Another approach to understanding mitochondrial DNA precursor metabolism was employed using a method that was first utilized by Wiegers *et al.* on HeLa cell RNA biosynthesis (100). Their observations demonstrated that HeLa cells used different precursor pools for the synthesis of ribosomal and messenger RNAs. The approach used in this thesis is similar to that of Wiegers *et al.* but measures instead deoxypyrimidine precursor utilization for nuclear and mitochondrial DNA biosynthesis. The method involves the presentation to cells of a radioactively labeled deoxypyrimidine precursor, namely uridine, for various times followed by measurement of the dCMP and dTMP specific activities in mitochondrial DNA as compared to nuclear DNA. Thus by providing an exogenous DNA precursor determination of its relative contribution to the dCMP and dTMP residues in the two different DNAs can be made. These experiments were done using mouse L-cells rather than HeLa cells for technical reasons described in the Results. The experiments suggest that the relative contribution of exogenous uridine to nuclear DNA dTMP and dCMP and mtDNA dTMP residues is approximately the same. However, under identical conditions mitochondrial dCMP residues are synthesized using twice as much exogenous uridine.

M. Present Work

The work described in this thesis investigates the level of HeLa cell mitochondrial dNTPs and rNTPs and how they vary as a result of antimetabolic perturbations using methotrexate and fluorodeoxyuridine. I have developed rapid mitochondrial dNTP and rNTP pool isolation methods and a means of quantitating these pools with respect to mtDNA yield. In addition I have investigated the utilization by mouse L-cells of an exogenous DNA precursor and the variation of mtDNA synthesis from nuclear DNA synthesis in this respect. The majority of the results of this work have been accepted for publication as a pair of papers in The Journal of Biological Chemistry (101,102). Experiments describing the development of dNTP extraction methodologies have been published (85).

II. METHODS

A. Materials

Radioactively labeled deoxyribonucleoside 5'-triphosphates obtained from New England Nuclear or ICN Chemical and Radioisotope Division were: [8-^{3}H] dATP, [CH_3-^{3}H] dTTP, [5-^{3}H] dCTP and [8-^{3}H] dGTP all between 2 and 30 Ci/mmole. Labeled deoxyribonucleosides obtained from New England Nuclear were [5-^{3}H] uridine (30 Ci/mmole), [6-^{3}H] uridine (25 Ci/mmole), and [5,6-^{3}H] uridine (40 Ci/mmole). Carrier-free (^{32}P) orthophosphate in 10 mM HCl and [α-^{32}P] dCTP (>600 Ci/mmole) were from New England Nuclear.

Methotrexate was from Sigma and 5-fluorodeoxyuridine was obtained from Hoffman-La Roche. Cytochrome C and Janus Green B were from Sigma Chemical Co. Unlabeled nucleotides were from Sigma Chemical Co., ICN (HPLC pure) or P-L Biochemicals, Inc. Highly purified *E. coli* DNA polymerase I (fraction VII, 5000 U/mg protein), T4 DNA polymerase, deoxyribonuclease I, and venom phosphodiesterase (*Crotalus adamanteus*) were purchased from Worthington Biochemical Corp. All restriction endonucleases were from Bethesda Research Laboratories. Proteinase K (Pronase Type XI) was from Sigma. Nitocellulose was from Schleicher and Schuell. Agarose was from Bio-Rad and ethidium bromide was from Sigma Chemical Co. Synthetic polynucleotides were from PL Biochemicals.

Cell growth media and trypsin were purchased from Grand Island Biological Co.

B. Cells and Media

Monolayers of HeLa F cells and mouse L-cells were grown at 37°C in Joklik-modified minimal essential medium (F-13) supplemented

with 10% fetal calf serum or 10% newborn calf serum (all sera were from Grand Island Biological Co. or Kansas City Biological Co.). HeLa S3 cells, adapted to spinner culture, were grown in the same medium. All media contained Penicillin at 100 units per ml and Streptomycin at 100 micrograms per ml; both were obtained from Grand Island Biological Co. When labeling cells with [^{32}P] orthophosphate, phosphate-free F-13 (Grand Island Biological Co.) was used and supplemented with 10% dialyzed fetal calf serum and with 10 mM HEPES at pH 7.3 to provide a buffer in place of phosphate. All sera were heated to 56°C for 20 hours prior to use. Cultures were routinely screened for mycoplasma by use of a fluorescent stain for DNA (Bioassay Systems). HeLa cells had a 18-24 hour doubling time and mouse L-cells doubled every 22-26 hours, depending on growth conditions.

C. Labeling Conditions for Cells

For experiments to determine mitochondrial utilization of uridine as a DNA precursor mouse L-cells were grown in 175 cm^2 flasks with 50 mls of phosphate-free F-13 with 10% newborn calf serum and 1 μCi/ml ^{32}P orthophosphate for at least 48 hours or approximately two cell doublings. The cells were then changed to the same medium supplemented with 200 μM [6-^{3}H] uridine or [5-^{3}H] uridine at 2.5 mCi/mmole. These cells were grown for up to 96 hours after the addition of [^{3}H] uridine with cells being harvested at the indicated times. The medium was changed every 12 hours to avoid depletion of either the [^{32}P] orthophosphate or [^{3}H] uridine. The cells were not allowed to reach confluency before harvesting, by seeding initially at the appropriate cell density.

D. Preparation of Extracts for dNTP and rNTP Analysis

Monolayer cells to be used for only whole cell extracts were grown in 100-mm Petri dishes. For extraction, medium was removed, 2.0 mls of ice-cold extraction solution was immediately added, and the cells were scraped from the plate into suspension with a rubber policeman. The plate was washed with another 1.0 ml of extraction solution and the wash combined with the first cell suspension.

HeLa spinner cells were used for dNTP quantitation of subcellular fractions. The cells were routinely grown to between $3x10^5$ and 10^6 cells/ml and harvested within these limits. One liter of cells was used for each drug treatment or control. Mitochondria, nuclei, and cytoplasm were rapidly isolated as described below and suspended with extraction solution. For mitochondria the final volume was 200 µl. For nuclei and cytoplasm the final volume was 400 µl.

Sixty percent methanol extraction was done at -20°C and cell or subcellular suspensions were stored overnight at -20°C. Insoluble material was removed by centrifugation at 27,000 x g for 20 minutes and the supernatant frozen in liquid nitrogen and lyophilized to dryness.

Extraction with 0.5 N perchloric acid was done at 0-4°C for 30 minutes. The acid precipitate was removed by centrifugation at 27,000 x g for 20 minutes. The supernatant was carefully neutralized with 1.5 N KOH until the residual phenol red from the cell growth medium turned pink. The pH was monitored and found always to be between seven and eight. The $KClO_4$ precipitate was removed by centrifugation at 27,000 x g for 20 minutes and the supernatant frozen in liquid nitrogen and lyophilized to dryness.

The combined methanol-perchloric acid extraction procedure was done by first extracting with methanol then resuspending the lyophilized material in at least 1.0 ml of 0.5 N perchloric acid and continuing the perchloric acid extraction procedure.

After the double extraction procedure, cytoplasmic extracts contained large amounts of sucrose that required removal before using in the enzymatic assay for dNTPs. This was accomplished by using a DEAE Sephadex column and a volatile buffer, triethylammonium bicarbonate (TEAB) at pH 8.0. TEAB is prepared by diluting triethylamine (Aldrich) to 1.0 M followed by bubbling CO_2 through the solution until the pH reaches 8.0. DEAE Sephadex A-25 (Pharmacia) was hydrated in 1.0 M TEAB and equilibrated with 50 mM TEAB. A 2 ml bed volume column was poured and 10 mls of 50 mM TEAB washed through. The sample (up to 5 mls) was loaded and nucleotides were allowed to bind to the DEAE. Another 10 mls of 50 mM TEAB was applied followed by 10 mls of 1.0 M TEAB. Nucleotides were detected in the first 3-4 mls following the 0.05 to 1.0 M TEAB step by monitoring absorbance at 260 nm. Fractions containing nucleotides were frozen in liquid nitrogen, lyophilized to dryness, and resuspended in 400 µl of water.

E. Enzymatic Assay for dNTPs

The enzymatic assay of dNTPs was essentially as described previously (85). Ten microliters of sample were added to 90 µl of a solution containing 50 mM Tris-HCl, pH 8.3, 5.0 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1.1 mM of the appropriate [^{3}H]-labeled dNTP (0.01 mCi/ml), 1.1 units/ml of E. coli DNA polymerase I, 0.035 mg/ml of an alternating copolymer template, either poly(dA-dT), poly(dI-dC), or poly(dG-dC) and 0.22 mg/ml of bovine serum albumin. The poly(dA-dT)

was used for assays of dATP and dTTP while poly(dI-dC) was used to assay dCTP and dGTP. As an example, the assay for dATP would measure the incorpora-tion of [^{3}H]-dTTP into poly(dA-dT). For each set of assays a standard curve was obtained from known amounts of the dNTP being assayed.

Samples and reaction components were mixed together at 0°C and reactions were initiated by transferring to a 37°C water bath. At the indicated times 25-µl aliquots were removed and spotted directly onto Whatman #3 or GFC 2.4-cm filter discs presoaked with 5% trichloroacetic acid (TCA), 1% sodium pyrophosphate (NaPPi). Filters were batch washed in the same TCA-NaPPi solution three times at 0-4°C, twice in 95% ethanol at 0-4°C, dried and counted in 7.5 mls toluene phosphor (105) for counting. Radioactivity was measured in a Beckman liquid scintillation counter.

Experiments using poly(dG-dC) as a template were done exactly as described above. When T4 DNA polymerase was used in the dNTP assay the reaction mixture consisted of 60 mM Tris, pH 8.6, 16 mM NH_4SO_4, 6.5 mM $MgCl_2$, 6.5 mM EDTA, 1 mM dithiothreitol, 0.063 mg/ml template DNA, 0.25 units of T4 DNA polymerase, 1.1 mM of [^{3}H]-labeled dNTP and either sample or dNTP standards.

F. Subcellular Fractionation and Isolation of Mitochondria

Spinner cells were pelleted by centrifugation at 37°C and monolayer cells were removed by trypsinization and pelleted by centrifugation. The cells were resuspended in SMED (250 mM sucrose, 20 mM morpholinopropane sulfonic acid (MOPS), pH 7.0, 3 mM EDTA and 2 mg/ml digitonin) at between $1x10^7$ and $5x10^7$ cells per ml. Lysis was accomplished by forcefully working the suspension through a 21 gauge

needle at least four times or until lysis was observed by phase contrast microscopy. All steps were done at 0-4°C.

Two different mitochondrial isolation procedures were used, both of which resemble those of Bogenhagen and Clayton (92). The first and most frequently used is analogous to their "no gradient" procedure. Nuclei are removed by one centrifugation at 400 x g (2,000 RPM in a SS34 rotor) for four minutes. The supernatant was checked for nuclear contamination by phase contrast microscopy and if free of nuclei was centrifuged at 27,000 x g for ten minutes. The pelleted mitochondria were then extracted for nucleotides or DNA. The time required from resuspension in SMED to the addition of extraction solution never exceeded 20 minutes.

The second method is similar to the "two step" procedure of Bogenhagen and Clayton (92). Cells were lysed as described above and layered (5 mls maximum) onto a discontinuous sucrose gradient consisting of 14 mls of 1.0 M sucrose, 10 mM Tris, pH 7.5, 5 mM EDTA, on top of 14 mls 1.5 M sucrose, 10 mM Tris, pH 7.5, 5 mM EDTA. Centrifugation was for 30 minutes at 22 krpm in a Beckman SW25.1 rotor. Mitochondria banded at the 1.0 to 1.5 M sucrose interface and were removed by fractionation. Mitochondrial fractions were pooled and diluted with two volumes 250 mM sucrose, 20 mM MOPS, pH 7.0, 5 mM EDTA and centrifuged at 27,000 x g for ten minutes to pellet the mitochondria. This pellet was either extracted for nucleotides or DNA as described elsewhere.

Cytochrome oxidase was assayed spectrophotometrically according to standard methods by determination of the decrease in absorbance at 550 nm in one ml of 5 mM KPO_4, pH 7.0, 0.08 mg/ml

reduced cytochrome c, with 10 to 20 µl of sample. Protein concentration was determined using the Coomassie Brilliant Blue method (106).

Mitochondria were stained with Janus Green B by first covering a clean, warm glass slide with a 200 µg/ml solution of Janus Green B (Sigma) in absolute ethanol, draining the slide and allowing the residue to completely dry. A few drops of a mitochondrial preparation were placed on the slide and kept at 37°C, 100% humidity for ten minutes. The slide was then covered with a coverslip, and examined and photographed at 400x or 1000x magnification.

G. DNA Purification from Whole Cells or Subcellular Fractions

For extraction of whole cell DNA, medium was removed from 150-mm dishes and 10 mls of 0°C 5 N perchloric acid was added immediately. The cell suspension was scraped off the dishes and placed on ice. An additional 10 mls of 0.5 N perchloric acid was used to rinse the dish and pooled with the first suspension. This was allowed to stay on ice for ten minutes, then centrifuged at 27,000 x g for ten minutes. The pellet was completely drained and 2.5 mls of 250 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS was added, warmed to 37°C and 500 µg of proteinase K added and incubated for two hours. An equal volume of phenol saturated with 100 mM Tris-HCl, pH 8.0 was added, mixed until homogeneous, and cooled to 0°C. The phases were separated by centrifugation at room temperature in a clinical centrifuge and the aqueous phase removed. Residual phenol in the aqueous phase was removed by ether extraction and the ether removed under vacuum. Fifty microliters of a heat treated (90°C, 10 minutes) mixture of RNAse A and RNAse T1 (10 mg/ml and 1 unit/ml, respectively) was added and allowed to incubate at 37°C for one hour. To ensure complete

degradation of RNA, 50 µl of a separate preparation of heat treated RNAse A (400 µg/ml) was added and the mixture incubated at 50°C for one hour. The solution was made 0.5% in SDS, and 200 µg of proteinase K was added, followed by incubation at 37°C for one hour. The mixture was then phenol and ether extracted as above, the aqueous phase made 200 mM in sodium acetate, and three volumes of 100% ethanol at -20°C was added. The mixture was gently mixed and allowed to stand at least one hour at -20°C. The ethanol precipitate was removed by centrifugation at 27,000 x g for 30 minutes, dried completely, and resuspended in a DNA storage buffer (10 mM Tris, 10 mM NaCl, 0.25 mM EDTA) or prepared for DNAse I digestion.

DNAs from subcellular fractions, either nuclei or mitochondria, were isolated in much the same way. The following is for fractions from two 175 cm^2 flasks of mouse L-cells, approximately $1x10^8$ cells. The cells were harvested with trypsin in versene, pelleted and washed once in 10 mls of phosphate buffered saline (PBS). The pelleted cells were resuspended in 10 mls of SMED, and mitochondria and nuclei were prepared by the differential centrifugation method. Nuclei were then suspended in 5 mls 25 mM Tris-HCl, 10 mM EDTA, 1% SDS, and mitochondria were suspended in 2 mls of the same buffer. The suspensions were made 100 µg/ml in proteinase K and allowed to incubate two hours at 37°C. This was followed by phenol-ether extraction, RNAse digestion, proteinase K digestion, and ethanol precipitation as described previously. The ethanol precipitate was dissolved in DNA storage buffer for hybridization or prepared for DNAse I digestion.

For extraction of mtDNA sequences from crude mtDNA preparations a hydridization method was employed. Cloned mouse L-cell mtDNA (104) was generously provided by David Clayton. The clone consisted of the entire mouse L-cell mitochondrial genome inserted into the Hae II site of plasmid pACYC (84,104,115). Milligram quantities of the plasmid were needed, so E. coli strain C600 was transformed with this plasmid, and plasmid DNA was isolated from several liters of amplified cells. The methods used are described in a later section.

One milligram of plasmid DNA was first cleaved with EcoRI in a four milliliter reaction, heat denatured (100°C, 10 minutes), quick chilled in an ethanol-ice bath, and 15 micrograms were spotted onto circular 24-mm nitrocellulose filters (Schleicher and Schuell, BA85). The filters were baked in a vacuum oven for two hours at 80°C and stored at 5°C until needed.

Hybridization followed the method of Wahl et al. (107) using the modifications of Thomas (108). The filters were prehybridized with one ml of prehybridization buffer in heat sealed plastic bags for at least six hours at 42°C followed by hybridization with heat denatured in vivo labeled mtDNA preparations for at least 24 hours at 42°C in 0.5 mls of hybridization buffer. The exact prehybridization and hybridization conditions are listed in a later section. The filters were then removed from their bags, washed three times in two times concentrated SSC, 0.1% SDS at room temperature and for five minutes each in 0.1 times concentrated SSC, 0.1% SDS at 45°C for 15 minutes each. The filters were then placed in one ml of distilled water and heated to 100°C for ten minutes to remove the hybridized DNA

from the filter. The denatured DNA was ethanol precipitated and resuspended in 40 µl 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and digested to oligonucleotides with DNAse I (see below).

H. Digestion of DNA to Deoxyribonucleoside Monophosphates

For experiments to determine radioactivity in individual deoxyribonucleoside monophosphates of DNA, the DNA was digested with DNAse I and followed with venom phosphodiesterase digestion to produce deoxyribonucleoside-5'-monophosphtes. Ethanol precipitated DNA was dissolved in 40 µl 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 25 µg DNAse I (10 mg/ml in 150 mM NaCl) was added and the mixture incubated at 37°C for one to two hours. Five microliters of 100 mM Tris, pH 10.2, was added and 25 µg of venom phosphodiesterase (10 mg/ml in 110 mM Tris-HCl, pH 8.5, 110 mM NaCl, 15 mM $MgCl_2$) added, followed by incubation at 37°C for two hours. The dNMPs were then prepared for HPLC.

I. Quantitation of mtDNA Using Southern Blots

To precisely quantitate the amount of mtDNA present in a mitochondrial preparation extracted for nucleotides it was necessary to develop the following method. A mitochondrial pellet from one liter of HeLa spinner cells ready to be extracted for nucleotides was vortexed and the volume of the slurry measured. An aliquot representing 1/100 of the total volume (approximately 10 µl) was removed and the remaining mitochondria were extracted for nucleotides (see above). To the 10 µl aliquot was added 40 µl of 100 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.5% SDS and 50 µg of proteinase K. Incubation at 37°C for one hour was followed by phenol-ether extraction and ethanol precipitation. The precipitate was resuspended in 10 µl of Bam Hl

reaction buffer, and one unit of Bam HI was added and the mixture incubated at 37°C for one hour. Restriction endonuclease addition was repeated once and an aliquot was loaded on a 0.5% horizontal agarose gel containing 0.5 µg/ml ethidium bromide.

Electrophoresis was carried out at 50 volts for 12 to 15 hours. The gel was photographed with a 300-nm ultraviolet transilluminator. The gel was then treated according to the method of Wahl et al. (107) to prepare the DNA for transfer to nitrocellulose. Briefly, the gel was treated twice for 15 minutes each with 0.25 N HCl, rinsed with water, treated twice for 15 minutes each in 0.5 N NaOH, 1 M NaCl, rinsed with water and treated twice for 30 minutes each in 0.5 M Tris-HCl, pH 7.4, 3 M NaCl to neutralize the gel. The gel was then placed on top of two sheets of Whatman 3MM paper saturated with 20X SSC and surrounded with plastic wrap. A sheet of nitrocellulose was then placed on top of the gel and two sheets of Whatman 3MM paper placed on top of it. A five cm stack of paper towels with an evenly distributed one kilogram weight served as the blot absorbant. Blotting was allowed to continue for at least four hours.

The nitrocellulose was baked in a vacuum oven at 80°C for two hours or at 70°C overnight, placed in a heat sealable plastic bag and prehybridized with the appropriate volume of 50% formamide, five times concentrated SSC (SSC is 150 mM NaCl, 15 mM sodium citrate), two times concentrated Denhardt's (109) reagent [Denhardt's reagent is 0.02% bovine serum albumin (Sigma), .02% polyvinyl pyrrolidone (Sigma, Mr 40,000), and .02% Ficoll (Pharmacia, Mr 400,000)], 50 mM sodium phosphate, pH 6.5, and 250 µg/ml of sonicated, denatured salmon sperm

DNA (Sigma). The prehybridization was carried out for 6-24 hours at 42°C. The prehybridization solution was removed by cutting one corner of the bag and draining for several minutes. The hybridization solution was identical to the prehybridization solution except that 50% (w/v) dextran sulfate was added to a final concentration of 10%. Hybridization was initiated by adding all but one ml of the hybridization buffer to the bag containing the nitrocellulose. The remaining one ml was heated to 65°C and mixed rapidly with a nick translated ^{32}P-labeled hybridization probe specific for mtDNA in 50 µl of distilled H_2O that had been denatured by heating at 95-100°C for ten minutes and quick chilled in an ethanol-ice bath. Hybridization was allowed to continue for between 16-24 hours at 42°C. Both the prehybridization and hybridization solutions were mixed by taping the plastic bag on the inside surface of a large mouth bottle that was rolled in a cell culture roller bottle apparatus. After the hybridization period the nitrocellulose was washed three times in two times concentrated SSC, 0.1% SDS for five minutes each at room temperature and then twice in 0.1 times concentrated SSC, 0.1% SDS for 30 minutes each at 40-50°C. The nitrocellulose was dried at room temperature and autoradiographed at -70°C with a Dupont Lightning plus intensifying screen and Kodak XR Omat X-ray film. The necessary exposure was never longer than 24 hours.

To quantitate the unknown mtDNA samples a series of mtDNA standards of known concentration were run in adjacent lanes on the same gel as the unknowns. After autoradiography the bands on the film corresponding to standards and unknowns were aligned with the nitrocellulose and the regions of hybridization cut out and counted in

the toluene-based liquid scintillation fluor. The standard counts were plotted versus the DNA quantity and the unknowns aligned to determine the amount of mtDNA in each extract.

J. Nick Translation

Nick translation was done by a modification of the method according to Rigby et al. (110). One microgram of DNA was dissolved in 50 µl of 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, 0.2 µM dATP, 0.2 µM dTTP, 0.2 µM dGTP, 200 µCi [α-^{32}P] dCTP (New England Nuclear, 400-600 Ci/mmole), 1 mg/ml BSA, 50 ng/ml DNAse I, and 100 units/ml DNA polymerase I. The reaction was allowed to continue for ten minutes at 15°C and heated at 65°C for ten minutes to inactivate the enzymes. Removal of the DNA from the reaction mixture was done with a 1.0 ml (bed volume) column (tuberculin syringe) of SP-Sephadex (Sigma SP-C50-120) equilibrated with 300 mM NaCl, 10 mM Na acetate, pH 5.0. The reaction mixture was loaded directly onto the column and eluted using the equilibration buffer, with two drop fractions being collected. DNA is eluted before unincorporated dCTP and protein with this column. Fractions containing DNA were pooled, made 100 µg/ml in salmon sperm DNA and nucleic acids were ethanol precipitated. This method routinely gave specific activities of between 1 and $2x10^8$ cpm per µg DNA.

K. Cloning of a HeLa mtDNA Hybridization Probe

The mtDNA hybridization probe that was used in the mtDNA quantitation method was a 1.1 kilobase (kb) fragment of HeLa mtDNA that was cloned into pBR322. This recombinant plasmid was constructed by the following methods. Supercoiled HeLa mtDNA was prepared by a modifi-cation of the method of Bogenhagen and Clayton (92). Pelleted

mitochondria (see above) from a one liter spinner culture were resuspended in 2 mls of 50 mM Tris, pH 7.5, 10 mM NaCl, and 10 mM EDTA and made 0.5% in SDS. Lysis was accomplished by heating at 37°C for five minutes. SDS was removed by adding 0.5 ml of a saturated CsCl solution and chilling on ice for at least ten minutes followed by centrifugation to remove the cesium dodecyl sulfate precipitate. The supernatant was removed and the volume adjusted to 6.25 ml with TSE (10 mM Tris, pH 7.6, 10 mM NaCl and 0.25 mM EDTA). Then 6.93 g of CsCl was added followed by 0.9 mls of 2 mg/ml ethidium bromide. The refractive index was adjusted to 1.3870 by adding either CsCl or TSE. Density equilibrium centrifugation was carried out in a Beckman 50 Ti rotor at 36,000 RPM for 48-60 hours at 20°C. Gradients were fractionated and analyzed for mtDNA by running 5 µl of each fraction on a horizontal 0.5% agarose gel. Those fractions containing mtDNA were pooled, dialyzed against two liters of TSE, ethanol precipitated, and resuspended in 20 µl of TSE. This preparation of mtDNA yielded supercoiled mtDNA with no visible contamination of cellular DNA or RNA as visualized on agarose gels.

To clone a fragment of mtDNA one microgram was digested with Hind III in a 20 µl reaction volume (all restriction endonuclease digestions were performed according to the supplier's recommendations), phenol extracted, ethanol precipitated and resuspended in 40 µl of T4 DNA ligase reaction buffer (66 mM Tris, pH 7.5, 6.6 mM $MgCl_2$, 10 mM dithiothreitol and 0.4 mM ATP). To this was added 0.5 µg of Hind III-digested pBR322 and 0.1 unit of T4 DNA ligase. Ligation was carried out at 14°C for at least 16 hours.

Transformation of bacterial cells with the above ligation mix was done by growing E. coli strain C600 to an $O.D._{590}$ of 0.65 in penassay broth, 50 mls of the cells were pelleted, washed once in 5 mls ice-cold 10 mM NaCl, resuspended in 5 mls of ice-cold 50 mM $CaCl_2$, incubated at 0°C for 15 minutes, pelleted and resuspended in 1 ml 50 mM $CaCl_2$.

The ligation mixture was made 50 mM $CaCl_2$, 10 mM Tris, pH 7.5, 10 mM $MgCl_2$and 10 mM NaCl in a volume of 100 µl of the $CaCl_2$-shocked cell suspension. This mixture was incubated at 0°C for 30 minutes, heated to 42°C for two minutes, cooled to room temperature for ten minutes, and diluted with concentrated nutrient broth to give a final broth concentration of 1X. This was incubated at 37°C for 30 minutes and aliquots were plated on Davis minimal agar plates with 50 µg/ml ampicillin and incubated at 37°C overnight. Ampicillin-resistant colonies were tested for tetracycline sensitivity by picking the colonies with sterile toothpicks and transferring to ampicillin (50 µg/ml) Davis plates containing tetracycline (25 µg/ml) plus ampicillin. Those colonies which proved to be ampicillin-resistant and tetracycline-sensitive were further analyzed by a colony DNA extraction procedure described below.

L. Plasmid Isolation

Large and small scale isolations of bacterial plasmid DNAs were done by identical methods developed in collaboration with Dr. James Summerton. The basic principle involves the selective precipitation of nucleic acids with the chaotropic agent sodium trichloroacetate (NaTCA). NaTCA is not available in reagent grade quality and therefore must be prepared from reagent grade

trichloroacetic acid (TCA) and NaOH (both from Baker) by the following method. A 454 g bottle of TCA was dissolved with 200 mls H_2O to give a near saturated solution of TCA. An equimolar amount of 10 N NaOH was dissolved in a minimal volume and placed on ice to cool. A separatory funnel was used to hold the NaOH solution which was slowly dripped into the TCA solution which had been placed in a bucket of wet ice. It was necessary to rapidly mix the dripping NaOH with the TCA by stirring the TCA solution with a magnetic stir bar. Temperature was critical and was constantly monitored and never allowed to exceed 40°C. The temperature was lowered by decreasing the drip rate of the NaOH. After the solutions were completely mixed the pH was checked and more NaOH or TCA (both in solution) were added to give a neutral pH. The volume is adjusted to give a final NaTCA concentration of 4.5 M. Before use, an equal volume of absolute ethanol was added and this solution was called EtOH-NaTCA.

For large scale bacterial plasmid isolation one liter of SM9 medium (see appendix A) supplemented with the appropriate antibiotics (ampicillin at 50 µg/ml or tetracycline at 25 µg/ml) was inoculated with 10 mls of an overnight culture grown in penassay broth also supplemented with antibiotics. Incubation was at 37°C with vigorous shaking. Uridine was added after 30 minutes to 100 µg/ml (103). The culture was grown to an $O.D._{590}$ of 0.5 to 0.7 then chloramphenicol was added to 100 µg/ml for amplification. The plasmid was amplified for 15 hours at 37°C with vigorous shaking. After amplification the culture was cooled to 0°C and the cells were pelleted by centrifugation, resuspended in 25 mls resuspension buffer (100 mM Tris-HCl, pH 8.0, 100 mM EDTA, 30% sucrose) at 0°C, repelleted by

centrifugation and resuspended in 25 mls resuspension buffer. Fifty microliters of diethyl pyrocarbonate was added to inhibit degradative enzymes, and the solution was kept at 0°C for five minutes. Then 6.25 mls of lysozyme (6 mg/ml made fresh in H_2O) were added and incubation carried out at 0°C for 20 minutes. Lysis was accomplished by adding 31.25 mls of lysis buffer (2.0 M NaCl, 10 mg/ml Brij 58, 4 mg/ml deoxycholate) at 0°C and gently mixing by inversion until the solution was homogeneous. Lysis was facilitated by allowing this solution to warm to room temperature for 20 minutes. Chromosomal DNA and cell wall debris were removed by centrifugation at 40,000 x g for 30 minutes at 0°C. The low-viscosity supernatant was removed by pipetting and four volumes of EtOH-NaTCA at 0°C was added to the supernatant and allowed to stand on ice for at least one hour but preferably overnight to selectively precipitate nucleic acids. The precipitate was collected by centrifugation for 30 minutes and as much supernatant as possible was removed by wiping the sides of the tube with a Kimwipe. The pellet was resuspended in 10 mls RNAse A (400 µg/ml in water, heated at 90°C for 10 minutes to inactivate DNAses), made 200 mM sodium acetate, pH 7.0, and incubated at 50°C for one hour. The DNA was ethanol precipitated, dried under vacuum, resuspended in 10 mls TSE and the absorbance at 260 nm was measured. This represents partially purified plasmid DNA, and further purification was by CsCl-EtBr density equilibrium centrifugaiton.

CsCl-EtBr fractionation of supercoiled plasmid DNA was performed on the partially purified plasmid preparation by the following method. Approximately 50 A_{260} units were run per gradient so the total number of gradients varied from prep to prep. The

following describes the preparation of two 10-ml gradients but if more were necessary they would be made up together and aliquoted into individual tubes. 100 A_{260} units of DNA were diluted to 14 mls with TSE and 13.9 g of CsCl were dissolved. The solution was wrapped in aluminum foil to prevent contact with light and 1.8 mls of 0.02% EtBr was added. The density was adjusted to 1.59 g/ml by measuring weight and volume, or the refractive index was adjusted to 1.3870 by adding dry CsCl or TSE. The solution was split into cellulose nitrate tubes, overlaid with mineral oil, and centrifuged at 36,000 RPM for 48 to 60 hours at 20°C in a Beckman 50 Ti rotor. The gradients were fractionated into 0.5-ml fractions and those fractions containing supercoiled DNA were pooled, the EtBr removed by extracting five times with CsCl-saturated isopropanol, diluted with 2.5 volumes of TSE and ethanol precipitated overnight at -20°C. The yield from this procedure varied depending on the plasmid being isolated and the idiosyncracies of the individual prep but ranged between one and four milligrams of supercoiled plasmid DNA per liter of starting bacterial culture.

Small scale isolation of plasmid DNA from a single colony of bacterial cells was performed by a scaled down procedure of the large scale isolation. A large colony of bacterial cells was scraped off the agar surface by using a wire loop. The cells were suspended in 50 µl of resuspension buffer by rapidly spinning the loop in the solution. The remaining steps are identical to the large scale procedure except for the 40,000 x g centrifugation after lysis. This was done at 30,000 x g for 30 minutes (20,000 RPM in SS34 rotor). The quantity of DNA recovered after precipitation from the RNAse step was generally enough to detect on two analytical gel tracks.

M. DNA Restriction Fragment Isolation

Preparative isolation of DNA restriction fragments was done by one of two methods. First, horizontal 1% low-melting-temperature agarose gels containing EtBr were used to separate restriction fragments and the desired fragments were cut out. The agarose was melted at 65°C for ten minutes and two volumes of 50 mM Tris, pH 8.0, 0.5 mM EDTA were added. The solution was kept at 37°C and twice extracted with phenol and three times with diethyl ether. The DNA was ethanol precipitated and resuspended in TSE. The second method used horizontal 0.5 to 1.0% agarose gels containing EtBr to separate the DNA fragments. The desired fragments were visualized by 300-nm UV light. A cut was made on the cathode side of the fragment with a razor blade and a piece of Whatman DE81 paper was placed into this slit. Electrophoresis was continued until the band had migrated onto the paper. The paper was removed, soaked in a small volume of 1.0 M NaCl, 50 mM Tris (7.6), 10 mM EDTA for at least ten minutes and removed by centrifugation of the liquid through a 25 gauge needle hole made in the bottom of a 0.4 ml micro test tube. The DNA was then ethanol precipitated and resuspended in a small volume of TSE. The first method gave approximately 40% yield whereas the second method gave 70 to 80% yield.

N. HPLC Separation of dNMPs and rNTPs

DNA samples enzymatically digested to dNMPs or nucleotide extracts were made 2.0% in sulfasalicylic acid and chilled to 0°C for ten minutes. The precipitated protein was removed by centrifugation for two mintues at 12,000 x g. The supernatant was collected and neutralized with 5 N NaOH. Mixtures of deoxyribonucleoside

monophosphates prepared in this way from digested DNAs were separated by high performance liquid chromatography on an anion exchange column (AX-10, 10 μm, 4.0 x 300 mm, Varian Instruments) by use of a Varian chromatography system. Solvent conditions were as follows: isocratic 10 mM $KPO_4^=$, pH 2.9, for one minute, 10-200 mM $KPO_4^=$, pH 2.9, gradient for ten minutes, isocratic 200 mM $KPO_4^=$, pH 2.9, for two minutes. The column was then rinsed with distilled water for two minutes and returned to initial conditions and equilibrated for five minutes. Flow rate was 2.0 ml per minute and column temperature was 30°C. Absorbance at 254 nm was recorded and 1.0 ml fractions were collected and radioactivity measured by adding 9 mls of a Triton X-100/xylene scintillation mixture and counting in a liquid scintillation spectrometer.

For separation of ribonucleoside triphosphates the same anion exchange column was used. Solvent conditions were isocratic 0.3 M $KPO_4^=$, pH 3.6, at 30°C and 2.0 ml per minute flow rate. Quantities were determined directly with a Varian CDS 401 data system that integrated and calculated the amount of rNTP in each peak.

When individual dNMPs contained both ^3H and ^{32}P, dual isotope counting was performed. The ^{32}P spill into the ^3H window was determined for each experiment and varied from 2.5 to 3.0 percent.

III. RESULTS

To determine the subcellular distribution of dNTPs in normal cells and antimetabolite treated cells three requirements had to be met; 1) a rapid method for cell lysis and subsequent fractionation of organelles, 2) an efficient means of extracting nucleotides from cells and subcellular fractions, and 3) an assay in the picomole range to quantitate the low levels of dNTPs found in cultured cells. Initial experiments were designed to meet these requirements.

A. Lysis of HeLa Cells with Digitonin

As described in the Introduction, digitonin-induced membrane solubilization has been shown to be a useful method for mitochondrial purification. Application of this lysis procedure to HeLa cells necessitated development of optimal lysis conditions and analysis of mitochondria prepared using this method. When HeLa S_3 cells were grown in suspension to between $3x10^5$ and 10^6 cells/ml pelleted and resuspended in 1/40 volume isotonic buffer containing 2 mg/ml digitonin (SMED), no lysis was seen as determined by 400X phase contrast microscopy. However, if shear forces were applied to this mixture lysis was observed. Sonication was employed but resulted in a lower yield of whole mitochondria. Dounce homogenization required too many strokes and too much time. The most desirable and quickest method was found to be a rapid and forceful working of the cell suspension through a 21 gauge needle mounted to an appropriately sized syringe. Careful observation of the cell suspension by phase contrast microscopy was the most reliable determinant of cell lysis. Under these conditions nuclear integrity was maintained, and in many cases I observed fragmented endoplasmic reticulum still attached to nuclei.

The optimal lysis conditions that yielded the lowest volume of lysate were determined by varying conditions and observation of the resultant lysate by phase contrast microscopy. The most critical variable was found to be the volume of SMED in which a given cell preparation was resuspended and not the concentration of digitonin used. Optimal concentrations of both HeLa cells and mouse L929 cells were found to be between $1x10^7$ and $4x10^7$ cells per milliliter in SMED at 2 mg/ml digitonin. At this concentration as few as four passages through a 21 gauge needle was sufficient for a gentle but complete (>95%) lysis.

Subcellular fractionation was performed by either sucrose gradient centrifugation or differential centrifugation as described in Methods. The sucrose gradient method resembles the "two step" procedure of Bogenhagen and Clayton and the differential centrifugation method resembles their "no gradient" procedure (92). Sucrose gradients yielded routinely cleaner preparations of mitochondria than those prepared by the differential centrifugation method. As shown in Figure 4 the cytochrome oxidase activity profile across one of these gradients shows a well defined peak at the sucrose step and little activity in the nuclear fractions at the bottom or in the upper fractions where mitochondrial membrane fragments would be found. DNA extracted from sucrose gradient-purified mitochondria and subjected to agarose gel electrophoresis showed little or no contamination with high molecular weight chromosomal DNA. Furthermore, phase contrast microscopy at 400X magnification of mitochondria prepared in this way also indicated undetectable nuclear contamination. Although the sucrose gradient-prepared mitochondria were relatively pure several disadvantages with respect to maintaining

Figure 4. Cytochrome oxidase activity across a one step sucrose gradient using a HeLa S3 cell digitonin lysis extract. This represents the enzyme activity from $5x10^8$ HeLa cells. Lysis gradient and centrifugation conditions are described in Methods.

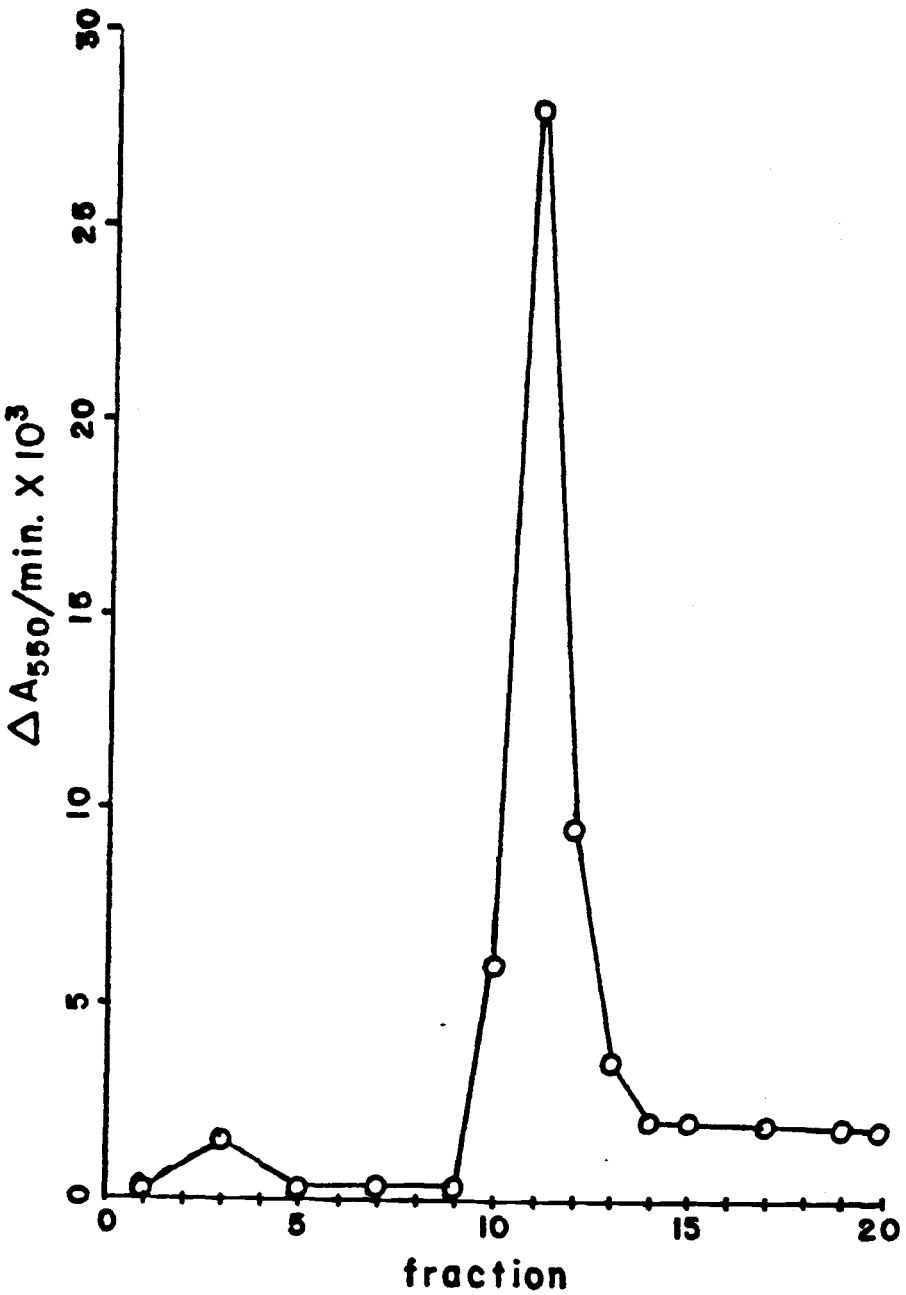

Figure 4. Cytochrome oxidase activity across a one step sucrose gradient using a HeLa S_3 cell digitonin lysis extract.

undisturbed intramitochondrial metabolites were possible. First, the time required to perform the isolation was over 60 minutes and second, the sucrose gradient subjected the mitochondria to sucrose concentrations of between 1.0 and 1.5 M, four to six times the isotonic sucrose value. Secondly, the longer time factor would allow intramitochondrial enzymes more time to alter deoxyribo-nucleotide levels, and the sucrose would have the effect of dehydrating the intramitochondrial matrix and again potentially altering deoxyribonucleotide levels. For these reasons the sucrose gradient procedure was often used for isolation of mitochondrial DNA and not for quantitation of intramitochondrial nucleotides.

The differential centrifugation procedure proved to be rapid and allowed recovery of all three subcellular fractions; nuclei, cytoplasm, and mitochondria. When mitochondrial DNA was purified from these preparations the yield was always higher compared to the sucrose gradient procedure but contamination with nuclear DNA was also higher. This is in agreement with Bogenhagen and Clayton, who reported a similar observation (92). Table II shows the relative purification of the mitochondrial marker enzyme cytochrome oxidase using the differential centrifugation method. The nuclear pellet had some cytochrome oxidase activity which is due to either trapped mitochondria or incomplete lysis. Because this method required from 15 to 20 minutes to perform and at no time subjected mitochondria to non-isotonic conditions it was employed when intramitochondrial deoxyribonucleotides were to be measured or when further purification of mitochondrial DNA could be performed.

Table II. CYTOCHROME OXIDASE ACTIVITY IN RAPIDLY PREPARED MITOCHONDRIAL FRACTIONS

Fraction	Units[a]
3.4×10^8 lysed cells	10.0
Mitochondrial pellet	6.10
Nuclear pellet	1.15

[a]Units are expressed as total absorbance unit change per minute at 550 nm for the entire preparation.

To determine the structural integrity of mitochondria prepared from digitonin-lysed HeLa or mouse L-cells, the mitochondrial-specific dye, Janus B Green, was used as a stain. This stain is reduced within the inner mitochondrial matrix to produce a blue green color. Mitochondria prepared by the differential centrifugation method were stained and, as shown in Figure 5, retained the reduced dye within the inner matrix. This observation, combined with the previously described sucrose gradient profile of cytochrome oxidase activity (Fig. 4) is evidence that the inner mitochondrial matrix is maintained intact during digitonin lysis.

B. Nucleotide Extraction and Assay of Whole Cell and Mitochondrial dNTPs

In order to measure dNTP levels in mitochondria, nucleotides must be rapidly and efficiently extracted from mitochondrial preparations so that mitochondrial or contaminating cellular enzymes do not alter dNTP levels during the process. Furthermore, the assay for dNTPs must be sensitive enough to detect the picomole levels of dNTPs present in mitochondrial extracts. For this reason it was necessary to scrupulously evaluate both the extraction procedure and the enzymatic assay used to quantitate dNTPs at the picomole level.

During analysis of 60% methanol extracts from intact HeLa cells addition of known amounts of dNTPs to these extracts resulted in a quantitative increase in assayed dNTPs (Fig. 6). Therefore, nothing in these extracts interferes with the enzymatic detection of dNTPs. When dNDP standards were added to these same extracts the apparent dNTP detected in the assay increased quantitatively, as though the dNDPs were actually dNTPs (Fig. 6). This strongly suggests the

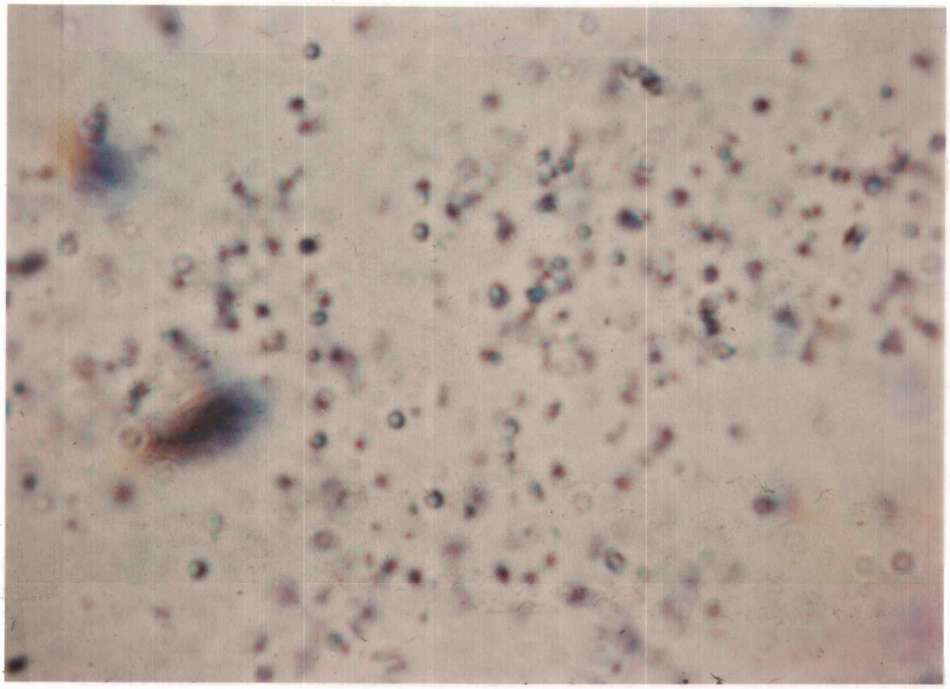

Figure 5. Mitochondria from digitonin lysed HeLa S_3 cells stained with Janus Green B. Staining conditions are described in The Methods.

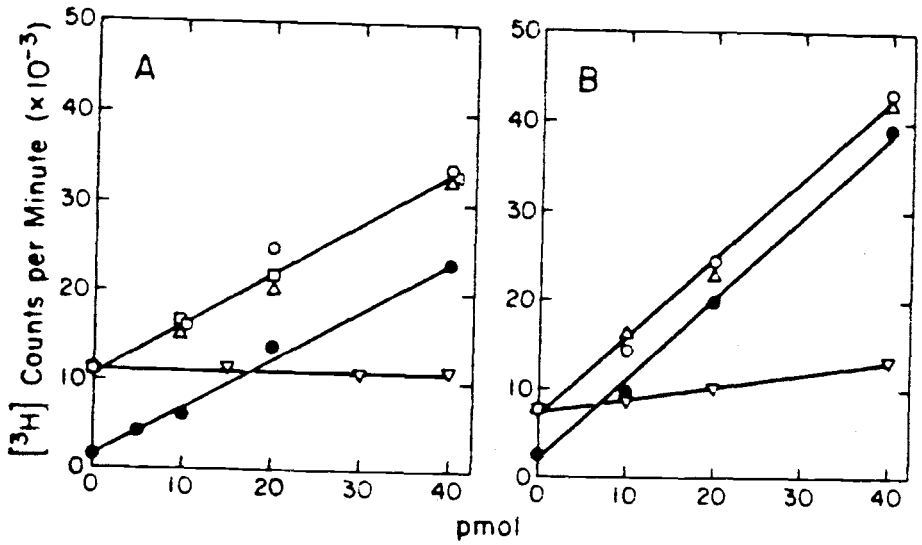

Figure 6. Enzymatic dNTP assays of deoxyribonucleotide standards added to 60% methanol extracts from HeLa cells. (A) dTTP assays of dTTP standards (●); or of dTTP (△), dTDP (○,□), or dTMP (▽) added to an extract. (B) dATP assays of dATP standards (●); or of dATP (△), dADP (○), or dAMP (▽) added to an extract. All extracts were diluted 40-fold before being assayed.

presence of nucleoside diphosphokinase activity in the 60% methanol extracts.

In experiments similar to those described above it was determined that 60% methanol also extracts dAMP, dCMP and dGMP kinases but not dTMP kinase. A spectrophotometric assay for dNMP kinase activity was too insensitive to measure the low level of dNMP kinase activity present in these extracts but nucleoside diphosphokinase can be detected (Table III).

Initial attempts at measuring dNTP levels in 60% methanol extracts of HeLa mitochondrial preparations indicated the presence of a very active nuclease (Fig. 7). A nuclease was also present in whole cell extracts but at a lower level. It appears the nuclease extracted from whole cells may be a mitochondrial specific nuclease since sucrose gradient purified mitochondria retain this nucleolytic activity at levels equivalent to whole cell extracts.

The net effect of these contaminating enzymatic activities is to increase the apparent dATP, dGTP and dCTP levels by nucleolytic degradation of the template and resynthesis of these dNTPs by the above-mentioned kinases. The resultant assays indicate varying amounts of ^{3}H-dNTP counter nucleotide incorporated into DNA as a function of time. As shown in Figure 7 the kinetics of these two processes change as the extracts are diluted. Furthermore, these data clearly show the effects of competing activities, kinases, on the enzymatic assay for dNTPs. No useful information on dNTP levels could be obtained with these results. Therefore, other extraction procedures were investigated.

Table III. NUCLEOSIDE DIPHOSPHOKINASE ACTIVITIES

Enzyme source	Activity[a]
HeLa cell lysate	5240 nmol/min/10^6 cells[a]
60% Methanol extract	1610 nmol/min/10^6 cells
Perchloric acid extract	<5 nmol/min/10^6 cells
E. coli DNA polymerase I	0.13 pmol/min/unit[b]

[a]Nucleoside diphosphokinase activity was assayed as described (85).

[b]A unit of *E. coli* DNA polymerase I incorporates 10 nmole of total nucleotide into DNA in 30 minutes at 37°C using activated DNA as a template.

Figure. 7. Nuclease activity in two different 60% methanol mitochondrial extracts. Both panels represent dATP enzymatic assays as described in The Methods. Extracts were assayed directly (●), or after being diluted 2x (△), 5x (□), or 10x (○).

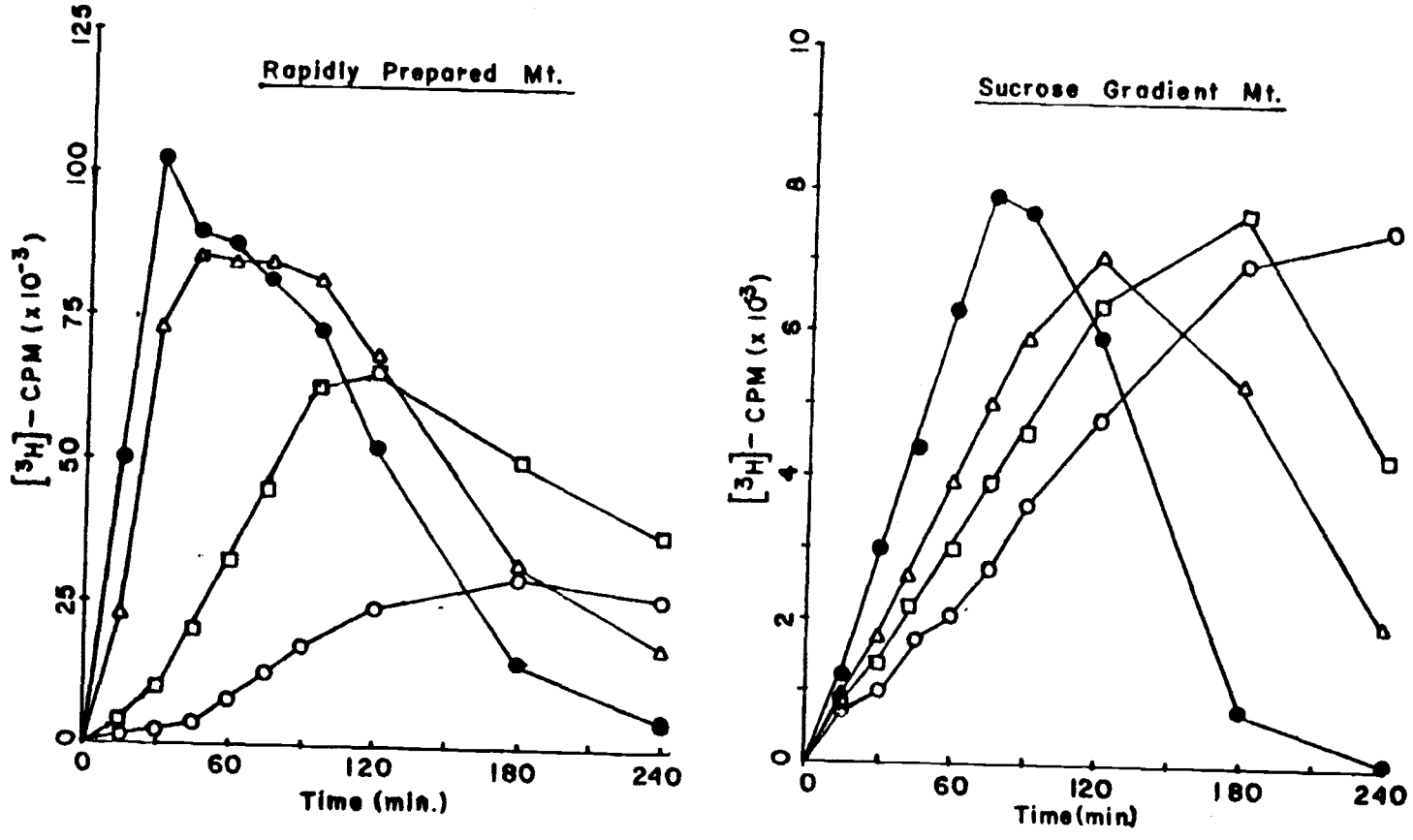

Figure 7. Nuclease activity in two different 60% methanol mitochondrial extracts.

When either mitochondrial or whole cell nucleotides were extracted with 0.5 N perchloric acid an interfering activity was also observed (Fig. 8). This activity prevented the enzymatic assay from reaching plateau values. No nucleoside diphosphokinase was detected in these acid extracts as it was in the 60% methanol extracts. Further characterization of the acid-extracted contaminating activity was not performed.

Because it was clear that methanol extraction leads to overestimation of dNTP pools and perchloric acid extraction does not allow the assay to reach plateau values, a two-step extraction procedure was used which eliminated the above-mentioned problems. Cells or mitochondria were first extracted with 60% methanol, then lyophilized to dryness and the dried material extracted with 0.5 N perchloric acid. These extracts were assayed for dATP and the time course was identical to that obtained with dATP standards (Fig. 9). This extraction procedure was used on all whole cell or subcellular preparations when either dNTPs or rNTPs were to be extracted.

C. Ribonucleoside Triphosphate Measurements

Quantitation of rNTPs was done with the same extracts used for the dNTP determinations. The assay is comparable to the procedures carried out in many other laboratories and is relatively easy due to the abundance of rNTPs. The assay involved HPLC analysis and is described in the Methods section. Figure 10 presents a chromatographic profile of a standard rNTP mixture and a profile obtained using a typical extract.

Figure 8. dATP assays of HeLa cell perchloric acid extracts. Whole cells and sucrose gradient mitochondria were extracted with 0.5 N perchloric acid and assayed as described in Methods.

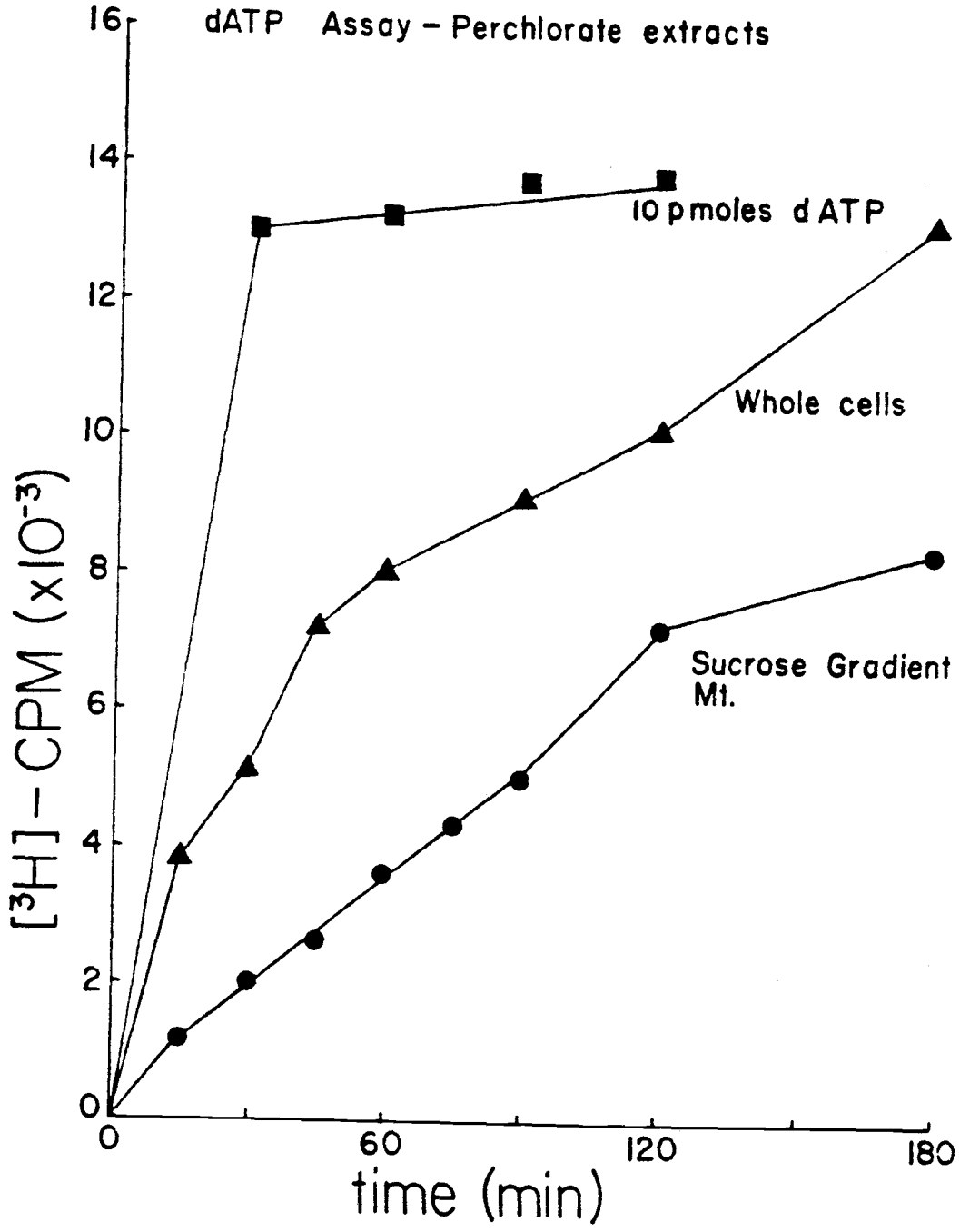

Figure 8. dATP assays of HeLa cell perchloric acid extracts.

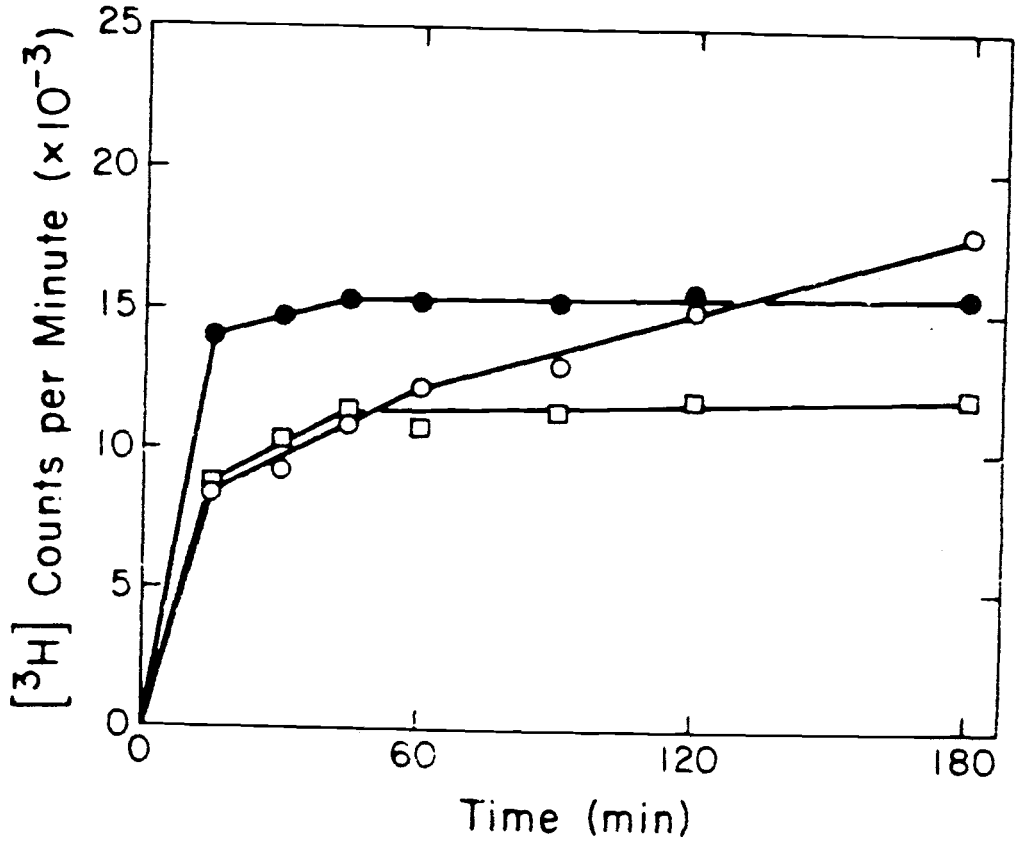

Figure 9. dATP assyas of Hela cell extracts. dATP assays of cells extracted with 0.5 N perchloric acid in 60% methanol (○), cells extracted first with 60% methanol followed by treatment with 0.5 N perchloric acid (□), and 10 pmoles of dATP standard (●). Details of extraction procedures are explained in the text and Methods.

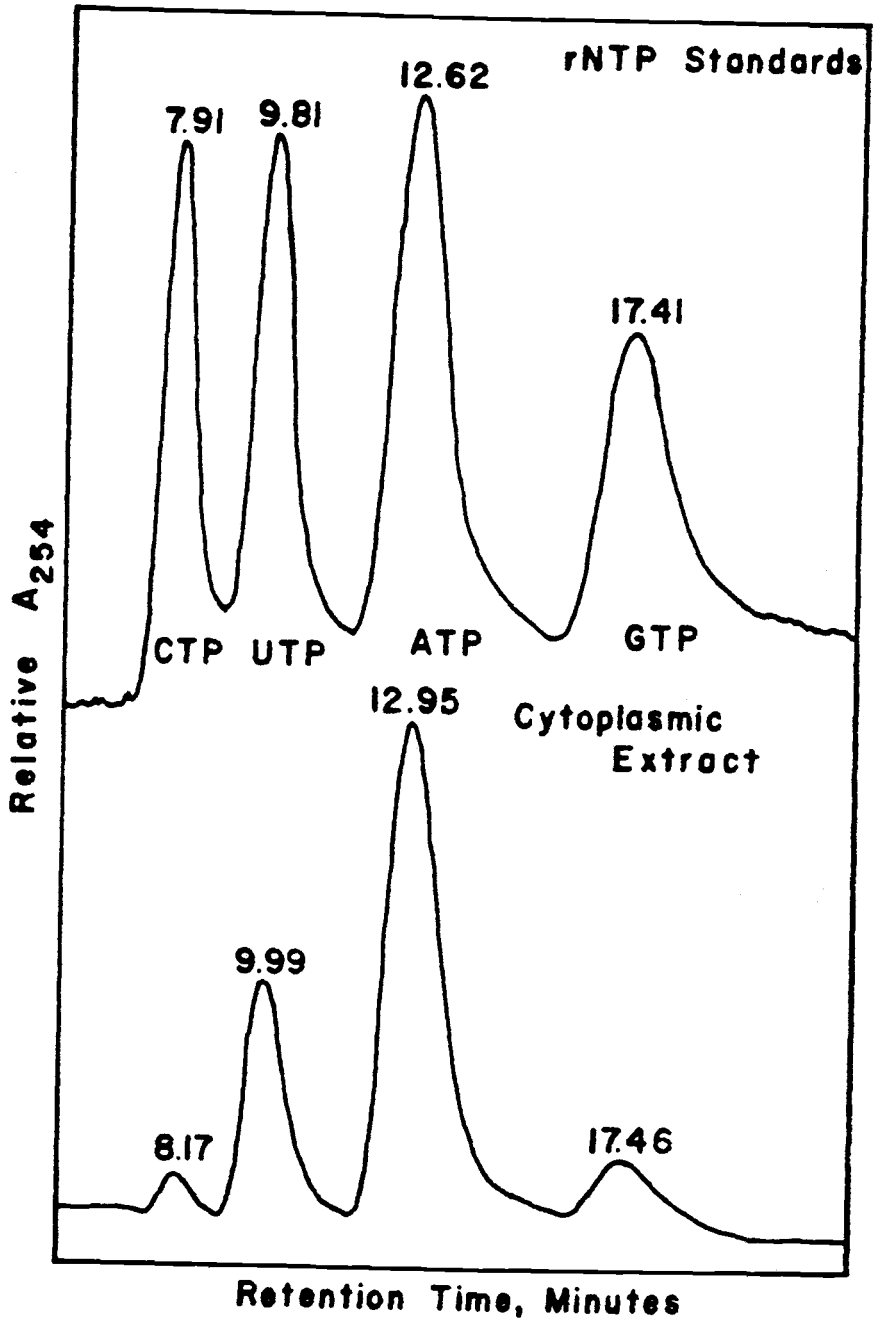

Figure. 10. Analysis of ribonucleoside triphosphate pools. Upper curve, HPLC elution profile of a standard rNTP mixture; lower curve, elution profile of a cytoplasmic extract from untreated HeLa cells. The numbers denote retention times for each peak.

D. dNTP Determinations from Whole HeLa Cells and Subcellular Fractions

To determine the efficiency of dNTP extraction when cells were fractionated it was necessary to compare the sum of the subcellular fractions with that of whole cells. A known number of cells was removed from the same batch of cells used for fractionation before lysis with digitonin and taken through the two step nucleotide extraction procedure along with the subcellular fractions. This was done with both drug treated and control cells. When normalized to picomoles dNTPs per 10^6 cells the comparison between the sum of the fractions and whole cells is very good for all four dNTPs from each of two separate isolations. This is shown in Figures 11 and 12. These data indicate that extensive destruction of subcellular pools did not occur during the subcellular fractionation procedure. Assayed levels of dNTPs present in mitochondrial extracts are not represented on the same scale as cytosol and nuclear levels since they are very small. The relative levels of dNTPs in nuclei and cytosol are variable between the two preparations shown in Figures 11 and 12 and probably reflect varying degrees of nuclear integrity and leakiness. However, the sum of nuclear and cytosolic pools closely approximates that for whole cells in both preparations. For these reasons the nuclear and cytosolic levels should be considered as representative of the nonmitochondrial or "cellular pools" of dNTPs. The effects of antimetabolites on dNTP levels shown in Figures 11 and 12 are discussed in a later section.

Figures 11 and 12. dNTP levels from antimetabolite treated HeLa S_3 isolated subcellular fractions. Pools were extracted after four hours of drug treatment. Cells or subcellular fractions were extracted from nucleotides and assayed for dNTPs according to the techniques described in the text. Untreated control, C; 1 μM methotrexate, ML; 10 μM methotrexate, MH; 1 μM fluorodeoxyuridine, F. These two figures are the results of two separate isolations. Each extract was assayed at least twice for each dNTP.

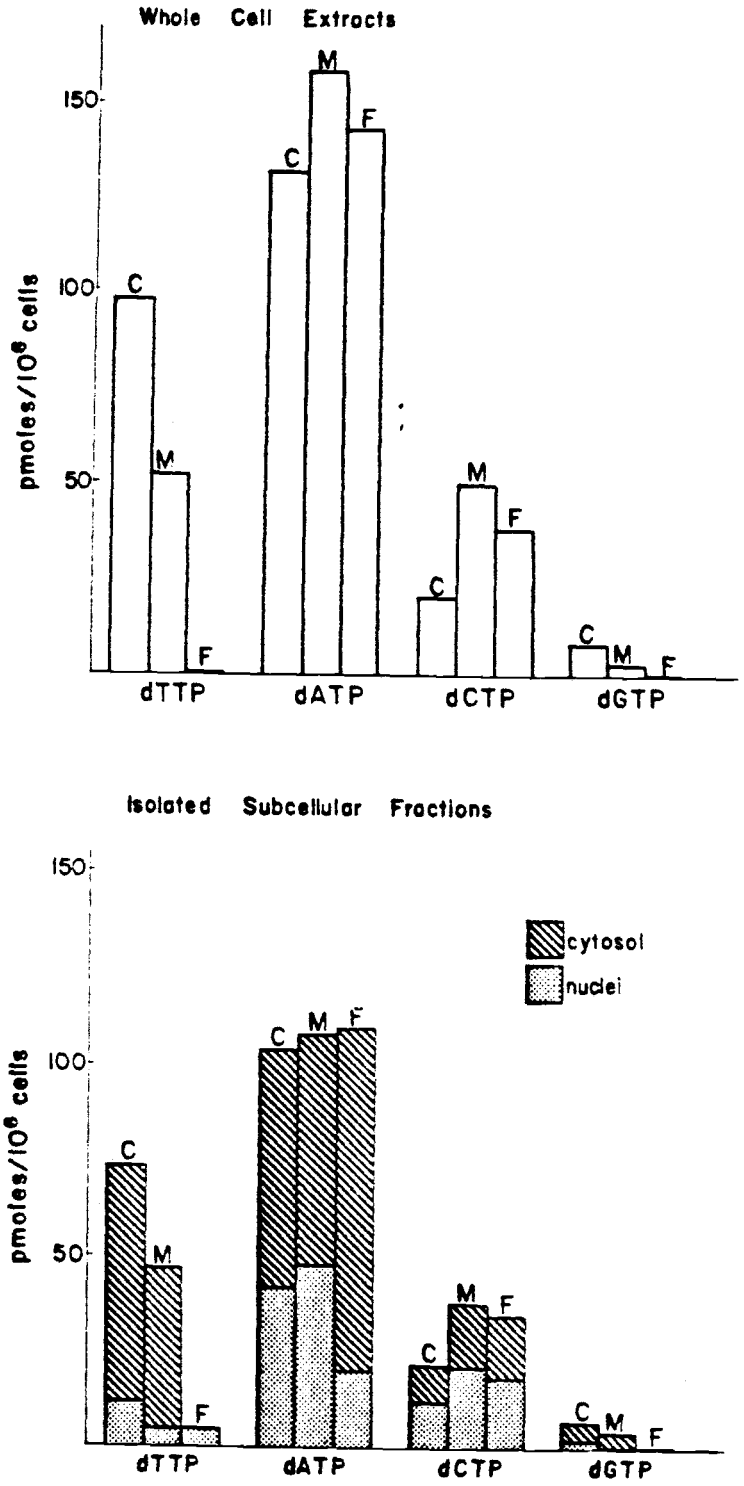

Figure 11. dNTP levels from antimetabolite treated HeLa S_3 isolated subcellular fractions

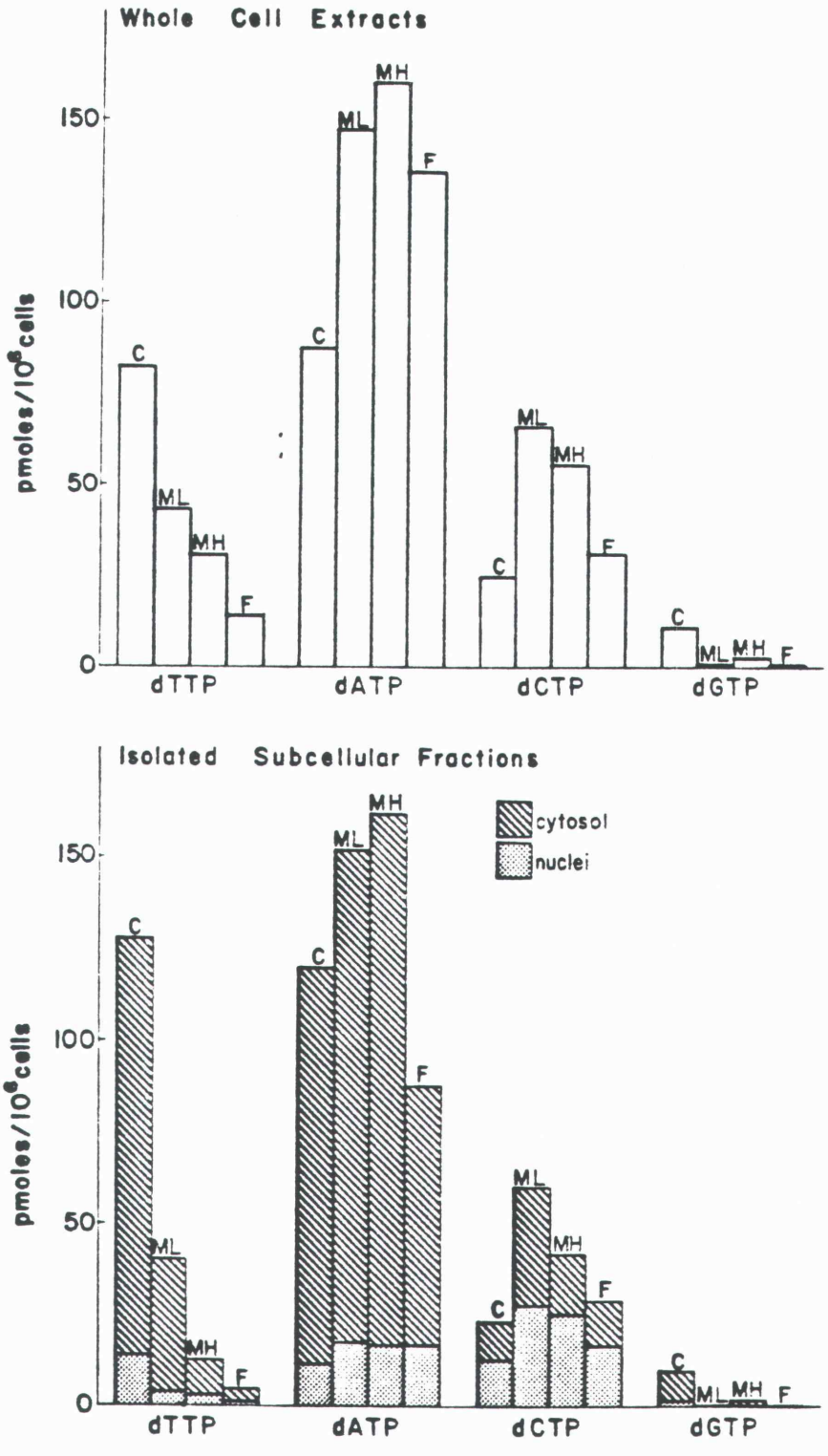

Figure 12. dNTP levels from antimetabolite treated HeLa S_3 isolated subcellular fractions.

E. dNTP Determinations From HeLa Mitochondria

Any isolation of mitochondria requires a method of quantitating the mitochondrial yield. This could be total protein, which in the case of rapidly prepared mitochondria would be misleading because of a lack of purity. A mitochondrial specific enzyme could be assayed but might vary due to metabolic stress imposed by the antimetabolites used in these experiments. The macromolecule chosen for quantitation in this work was mtDNA. The method chosen to determine mtDNA content was to remove 1% of the mitochondrial preparation before nucleotide extraction, purify the DNA and quantitate the mitochondrial specific DNA using the Southern blot technique. Figure 13 shows a photograph of an ethidium bromide-stained agarose gel with mtDNA standards and DNA isolated from rapidly purified mitochondria and cleaved with Bam HI which, cleaves mtDNA only once. A hydridization probe specific for HeLa mtDNA was cloned from Hind III digested mtDNA into pBR322 by standard techniques outlined in The Methods. Since I and others (111,112) have had negative results when attempting to clone HeLa mtDNA it was necessary to show definitively that the cloned DNA was a mtDNA fragment. Figure 14 is a restriction endonuclease map of HeLa mtDNA (113). The portion of mtDNA cloned is the 1.1 kb Hind III fragment four kb clockwise from the origin of replication. The pBR322 derived plasmid containing this fragment was named pRB-12. Figure 15 is a Southern blot using this fragment as a ^{32}P-labeled hybridization probe. The blot clearly shows this probe hybridizing with both supercoiled and nicked circular mtDNA. The cloned 1.1 kb Hind III fragment was nick translated and used to probe the gel shown in Figure 13 using Southern blot

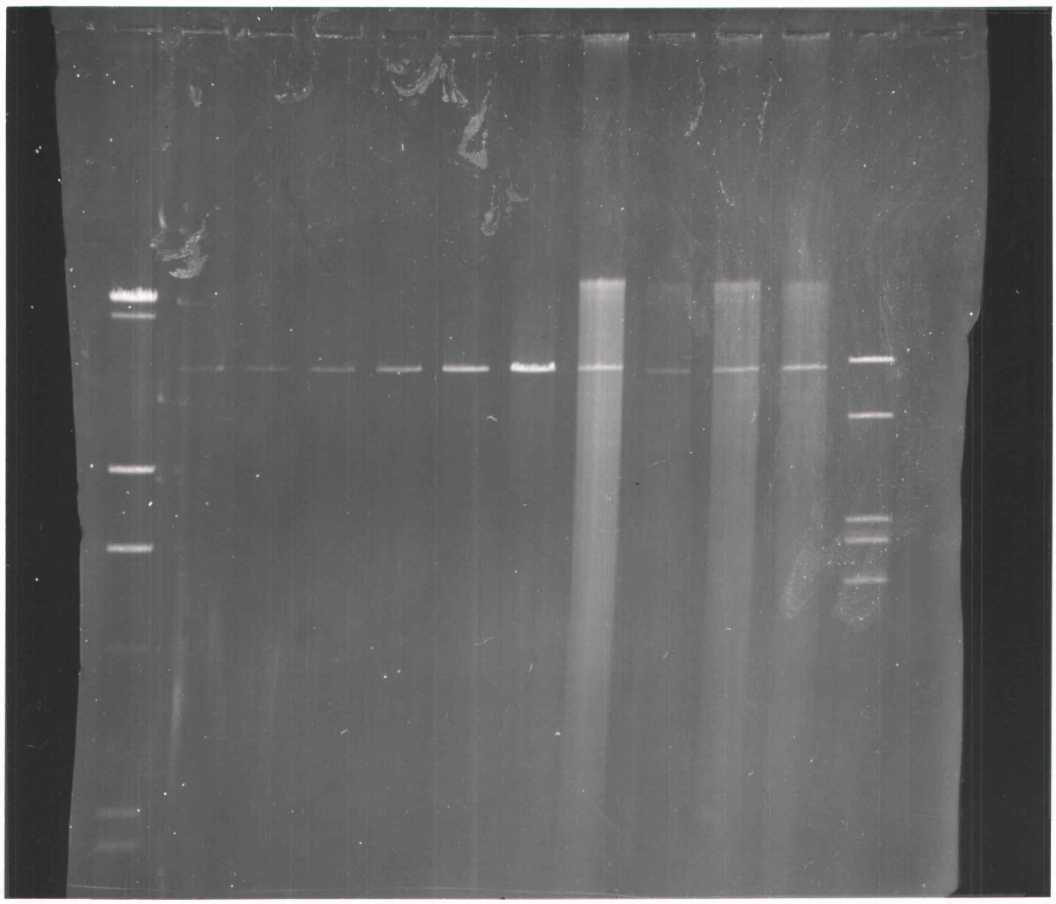

Figure 13. Agarose gel (0.7%) of mtDNA standards and unknowns. Electrophoretic conditions were 2 volts per cm for 15 hours. Sample descriptions are described in the legend to Figure 16.

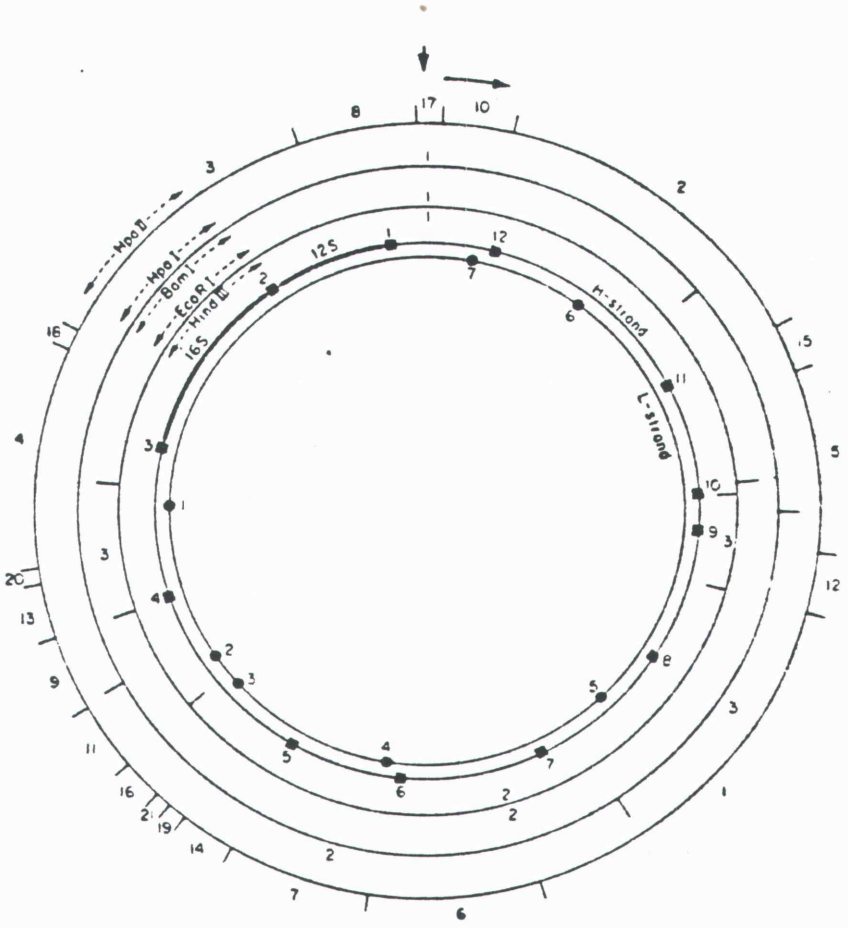

Figure 14. Restriction endonuclease cleavage map of HeLa cell mtDNA. From Ojala and Attardi (113).

Figure 15. Southern blot analysis of cloned HeLa cell mtDNA. DNA was electrophoresed on a 0.5% agarose gel at 100 V for 6 hours and then blotted via the Southern transfer procedure and hybridized to a ^{32}P-labeled 1.1 kb fragment of cloned mtDNA. Lane 1, 0.1 µg of partially purified HeLa mtDNA; lane 2, 0.1 µg of pRB-12 DNA; lane 5, 0.1 µg of 1.1 kb Hind III insert obtained from pRB-12. In lanes 1 and 2 the lower bands correspond to form I supercoiled DNAs and the upper bands correspond to form II nicked circular DNAs. The numbers at the sides of this autoradiogram were derived from the location of Hind III-digested wild type lambda marker DNA fragments (in kilobases) run in an adjacent lane.

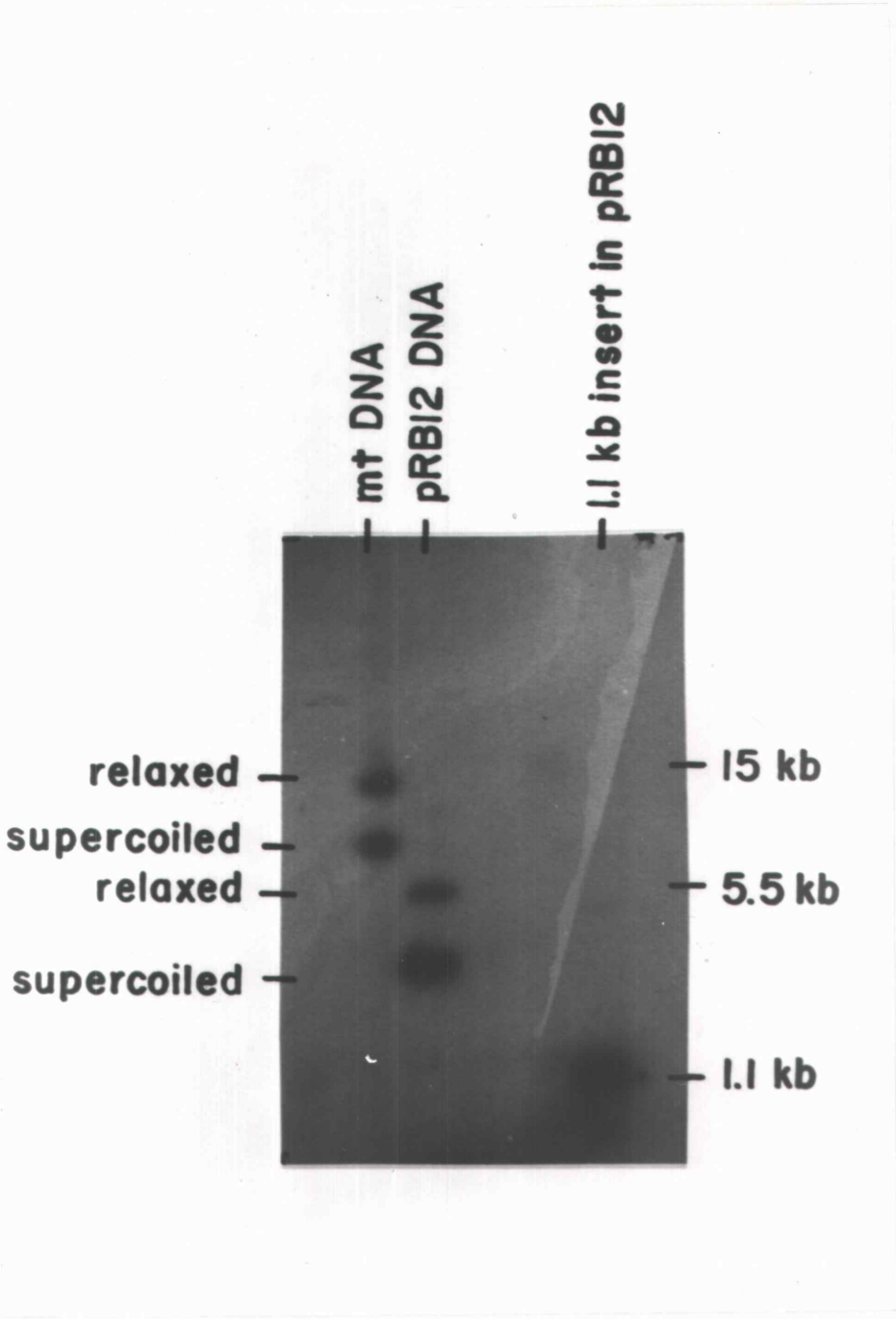

Figure 15. Southern blot analysis of cloned HeLa cell mtDNA.

hybridization. Figure 16 is an autoradiogram of this blot and demonstrates the hybridization to unknown mtDNA samples is within the standards. The nitrocellulose regions corresponding to the regions of hybridization were cut out and radioactivity was determined by liquid scintillation spectrometry. Figure 17 is a graphic representation of these data and clearly indicates the excellent linearity of the mtDNA standards. The counts hybridized to the unknowns were aligned and the corresponding nanograms of DNA determined. By use of this method a precise quantitation of mtDNA can be used to normalize dNTPs in mitochondrial extracts.

According to the work of Bogenhagen and Clayton (92) a value of 0.15 μg per 10^6 HeLa cells was used as the total amount of mitochondrial DNA present in HeLa cells. When the assayed levels of mitochondrial dNTPs and rNTPs are multiplied by a factor reflecting the yield of mtDNA, normalized values are obtained. By use of this rationale, Table IV presents picomolar dNTP and rNTP values for mitochondrial, cytoplasmic, and nuclear fractions from 10^6 untreated HeLa cells. Consistent with the results of others, the rNTP pools are up to 100 fold greater in size (2) and guanine nucleoside triphosphates are comparatively much lower. As expected, ATP is proportionately much more concentrated in mitochondrial fractions when compared to the other nucleoside triphosphates due to the fact that mitochondria represent the site of intracellular ATP synthesis. The observation that most of the cellular dNTP content is present in the cytoplasm is of interest.

As noted previously with respect to Figures 11 and 12, the possibility of leakage of dNTPs from nuclei into the cytoplasm cannot

Figure 16. Southern blot analysis of mtDNA from rapidly prepared mitochondria. Lanes 1 through 6 are known amounts of highly purified Bam H1 cleaved mtDNA. The amounts in lanes 1 through 6 are 1.25, 1.25, 2.5, 5.0, 10, and 20 nanograms of DNA, respectively; lanes 7 through 9 are Bam H1 cleaved DNAs from rapidly prepared mitochondria. DNA was electrophoresed on a 0.7% agarose gel for 15 hours.

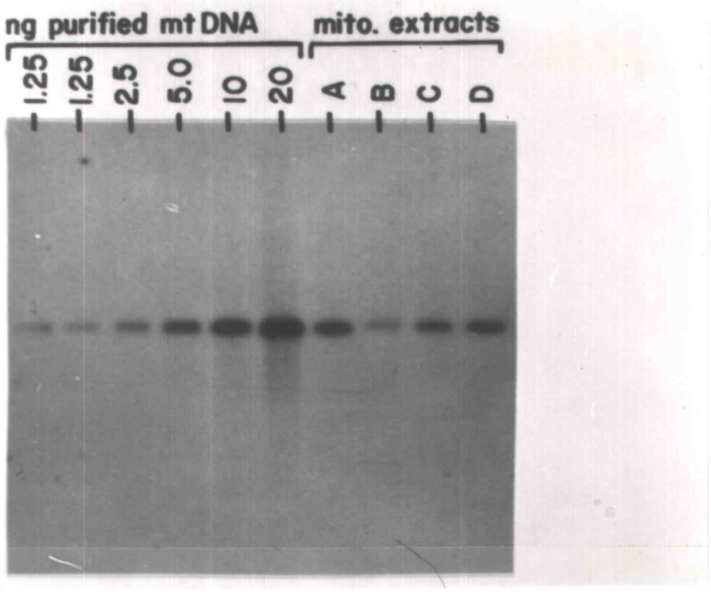

Figure 16. Southern blot analysis of mtDNA from rapidly prepared mitochondria.

Figure 17. CPM hybridized to known amounts of mtDNA. Known amounts of highly purified mtDNA along with unknown amounts of rapidly prepared mtDNA from mitochondrial preparations used for dNTP extractions were electrophoresed on a 0.7% agarose gel. The DNA was transferred to nitocellulose and probed with a ^{32}P-labeled nick-translated mtDNA hybridization probe. The nitrocellulose spot corresponding to each mtDNA sample was cut out and readioactivity determined by liquid scintillation spectrometry. Details are given in Methods. mtDNA standards, ●; unknown mtDNA samples, ↓.

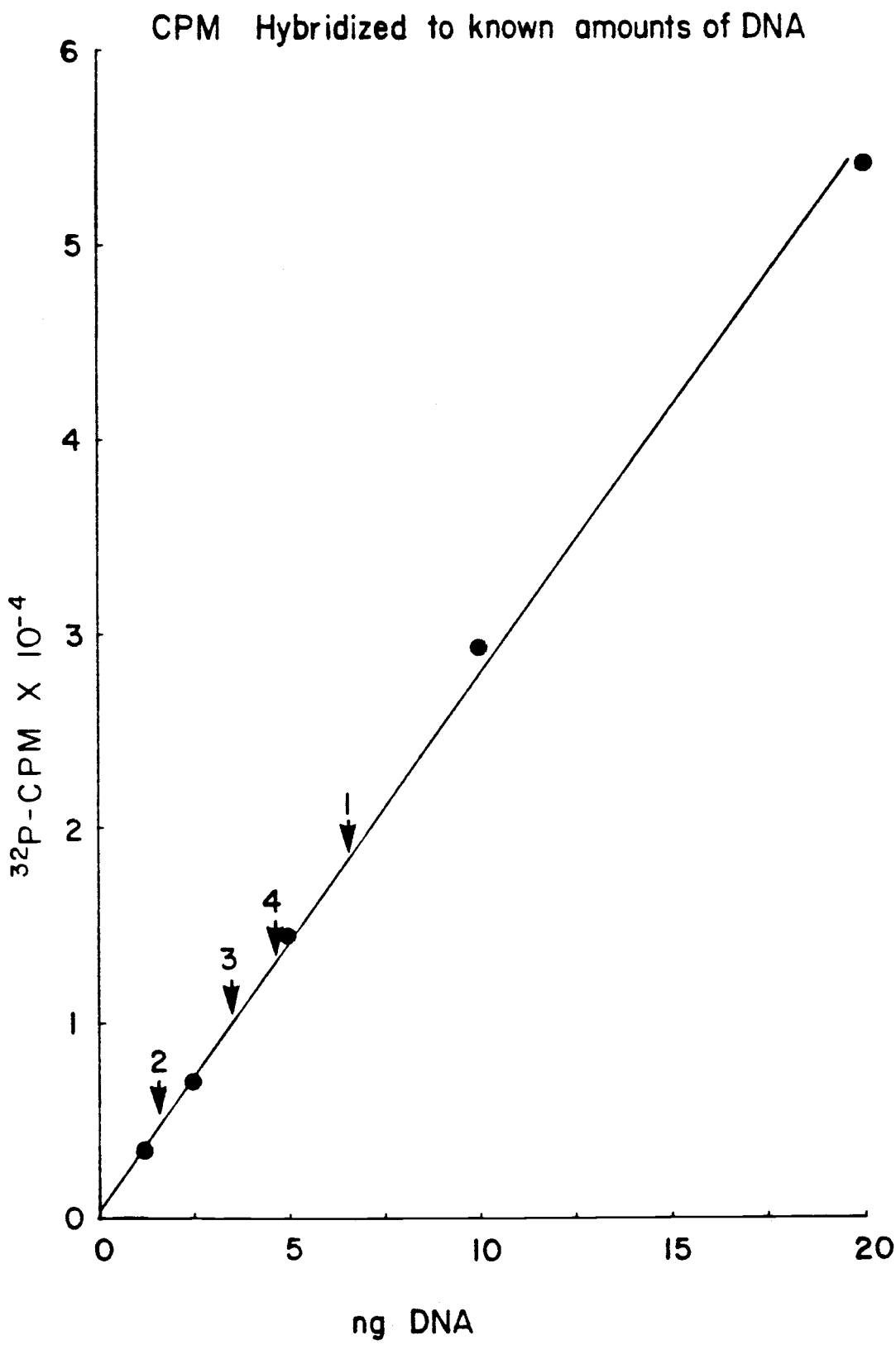

Figure 17. CPM hybridized to known amounts of mtDNA.

be ruled out. This is due to the known permeability of the nuclear membrane and the limitations of the cellular fractionation method used. However, evidence that some semblance of actual nuclear nucleoside triphosphate pools are maintained is apparent from the calculation of cytoplasmic to nuclear nucleoside triphosphate ratios presented in Table IV. When the four dNTP ratios were compared, only dCTP was observed to be partitioned about equally between the nuclear and cytoplasmic fractions. Had the large cytoplasmic pools resulted from dNTP leakage out of nuclei during isolation, then most of the dCTP would have been expected to be in the cytoplasmic fractions as well.

The mitochondrial dNTP pools are somewhat larger than one might expect, based upon published data relating cellular dNTP pools to nuclear DNA content. HeLa cells contain about 16 μg of DNA per 10^6 cells (114) and about 0.15 μg of mtDNA per 10^6 cells (92). Based upon these values the cellular dNTP pools from Figure 17 can be calculated, in picomoles per microgram cellular DNA as follows: dATP, 7.5; dTTP, 8.0; dGTP, 0.6; dCTP, 1.4. Corresponding values for the mitochondrial pools are dATP, 11.3; dTTP, 18.0; dGTP, 6.7; dCTP, 16.0.

Using the mitochondrial dNTP pool sizes and whole cell dNTP pool sizes, one can calculation mitochondrial dNTP pools as a percentage of whole cell dNTP pools. Figure 18 presents these data from untreated as well as antimetabolite treated HeLa cells. The effects of antimetabolites on mitochondrial pools is described in more detail in the following section.

Table IV. NUCLEOSIDE TRIPHOSPHATE DISTRIBUTION IN SUBCELLULAR FRACTIONS FROM GROWING HELA CELLS

Nucleotide	Pool Size, pmoles/cell x 10^6			Ratio
	Cytoplasmic	Nuclear	Mitochondrial	Cytoplasmic/Nuclear
dATP	108	12	1.7	9.0
dTTP	114	14	2.7	8.1
dGTP	8	1.8	1.0	4.4
dCTP	10	13	2.4	0.8
ATP	2920	200	605	14.6
UTP	1460	110	150	13.3
GTP	660	30	100	22.0
CTP	330	40	60	8.3

Figure 18. HeLa mitochondrial dNTP pools as percent of whole cell dNTP pools. Whole cells or mitochondria were isolated from untreated and drug treated cells, nucleotides extracted and dNTPs assayed enzymatically. In each quadrant the bars represent from left to right: untreated control cells, one micromolar methotrexate, ten micromolar methotrexate, and one micromolar fluorodeoxyuridine. Values along the ordinant are percentages.

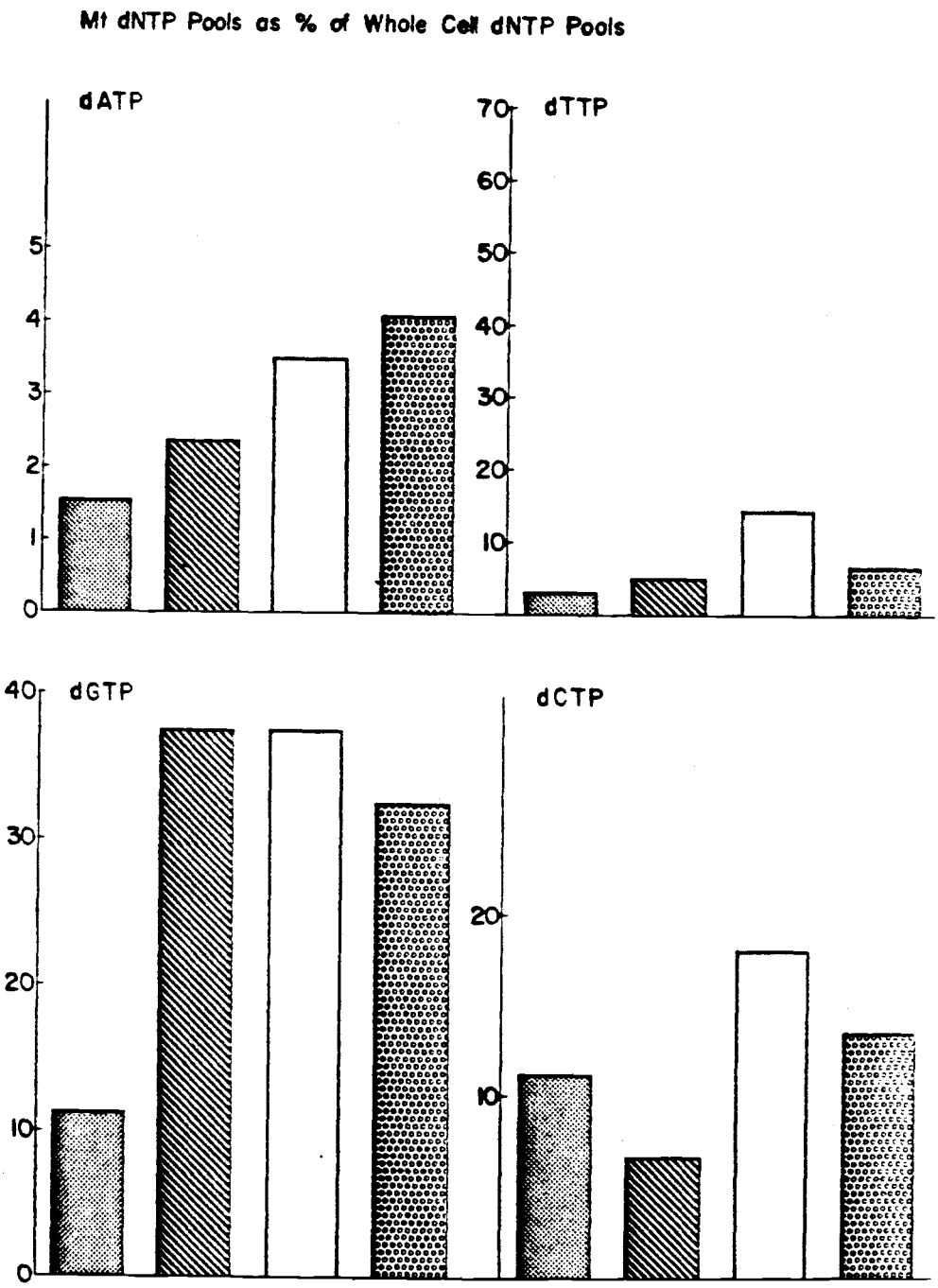

Figure 18. HeLa mitochondrial dNTP pools as percent of whole cell dNTP pools.

F. Effects of Antimetabolites

To develop a basis for study of antimetabolite effects on subcellular fractions, whole cell dNTP pools were measured after treatment with methotrexate and fluorodeoxyuridine. Shown in Figures 11 and 12 are the effects of four hours' treatment with two concentrations of methotrexate, one and ten micromolar, and one micromolar fluorodeoxyuridine on whole cell and cellular pools and dNTPs. An important comparison to make involves the relative levels of each dNTP as a function of drug treatment. Methotrexate at one and ten micromolar decreases dTTP levels substantially in both whole cells and in cellular pools with ten micromolar having the most pronounced effect, as would be expected due to the inhibition of thymidylate biosynthesis. Fluorodeoxyuridine at one micromolar has an even more dramatic effect than ten micromolar methotrexate on dTTP levels in both whole cells and the fractions. Methotrexate at one and ten micromolar appeared to have a positive effect on dATP levels even though de novo purine nucleotide biosynthesis were inhibited by methotrexate. The dATP pools from fluorodeoxyuridine-treated cells were unchanged in the first preparation but higher than the control in whole cells and lower in the cytosol and nuclear fractions from the second preparation. Levels of dCTP resembled dATP because methotrexate increased the size of the pools and fluorodeoxy-uridine caused only a slight increase. The drug effects on dCTP were nearly identical when the whole cell extracts were compared with the sum of the subcellular fractions. Levels of dGTP were extremely small in comparison to the dNTPs and virtually disappear in drug-treated cells.

One of the primary objectives of this thesis is the demonstration of antimetabolite effects on mitochondrial dNTP levels. This is probably best shown in Figure 18 where the effects of two concentrations of methotrexate, one and ten micromolar, and one micromolar fluorodeoxyuridine are presented. These data are calculated and graphed as a percent of total cellular dNTPs. In all the drug-treated cells the mitochondrial pools remain at control levels or above. Of interest are the relative sizes of the dGTP pools when treated with both methotrexate and fluorodeoxyuridine. It should be noted that because the whole cell levels of dGTP are very low before drug treatment, the mitochondrial pools appear exaggerated in drug-treated cells when presented in this way. To avoid this bias it is necessary to analyze the absolute levels of each nucleotide as a function of drug treatment. This is done in the following section.

G. dNTP Pools as a Function of Methotrexate Concentration

The difference in dNTP pools between mitochondria and whole cells treated with two concentrations of methotrexate are shown in Figures 19 and 20. The data in Figure 19 show the absolute levels of dNTPs for 10^6 cell equivalents of mtDNA, 0.15 µg mtDNA. Methotrexate at one micromolar increases or has little effect on all four mitochondrial dNTP pools. At ten micromolar, methotrexate causes a large increase in the dCTP level and significantly higher levels of dTTP and dATP. The mitochondrial dGTP level is not significantly affected by methotrexate at either concentration. The data in Figure 20 are from whole cells. As expected methotrexate at ten micromolar lowers dTTP levels in whole cells to 35% of control levels. Both dATP

Figure 19. Changes in the levels of HeLa mitochondrial dNTPs as a function of methotrexate concentration. Cells were treated for four hours with the indicated methotrexate concentrations, mitochondria were isolated, nucleotides were extracted and assayed enzymatically. The picomole values have been normalized to 0.15 µg of mtDNA which is equal to 10^6 cell equivalents of mitochondria (92).

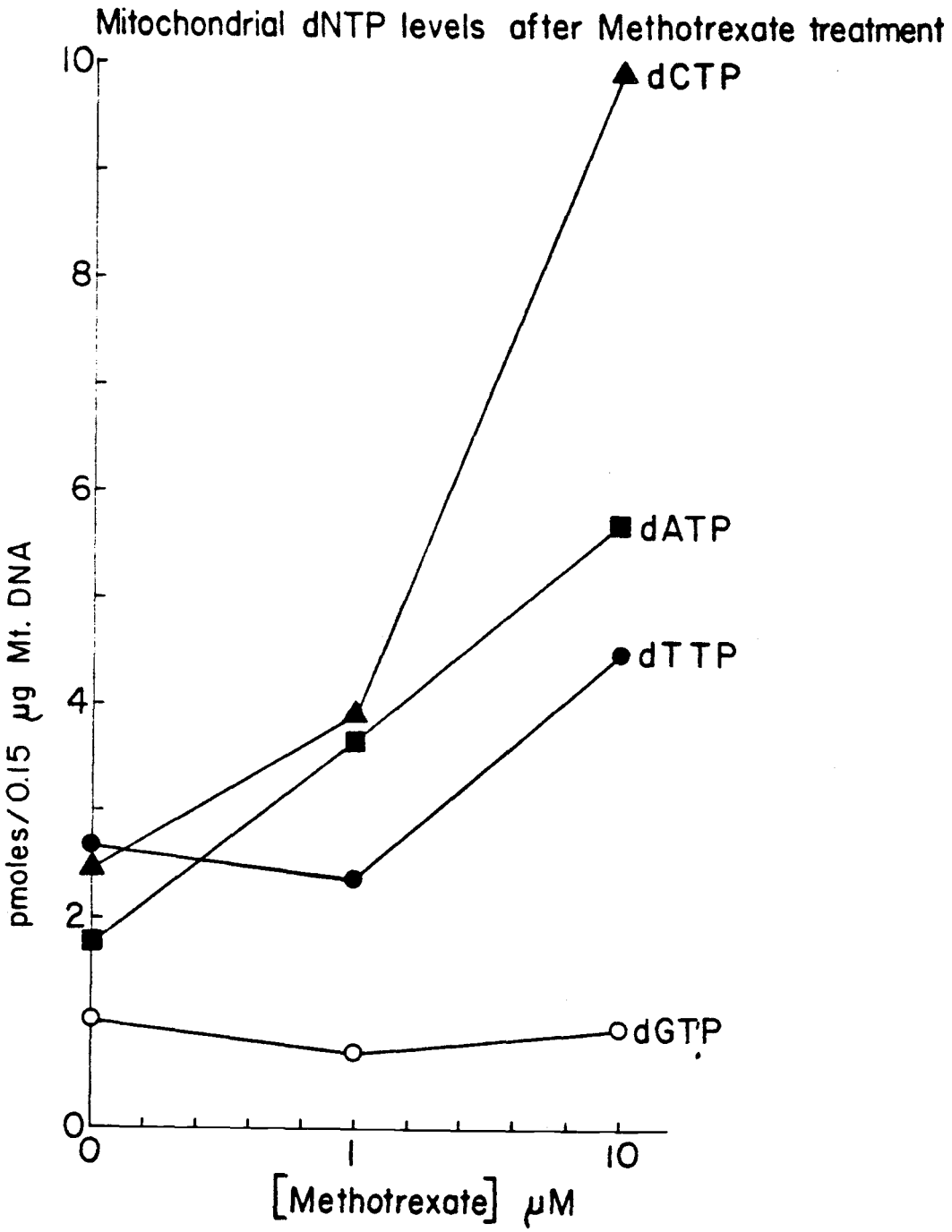

Figure 19. Changes in the levels of HeLa mitochondrial dNTPs as a function of methotexate concentration.

Figure 20. Changes in the levels of HeLa cell dNTPs as a function of methotrexate concentration. Cells were treated for four hours with the indicated methotrexate concentrations, nucleotides were extracted and assayed for dNTPs enzymatically.

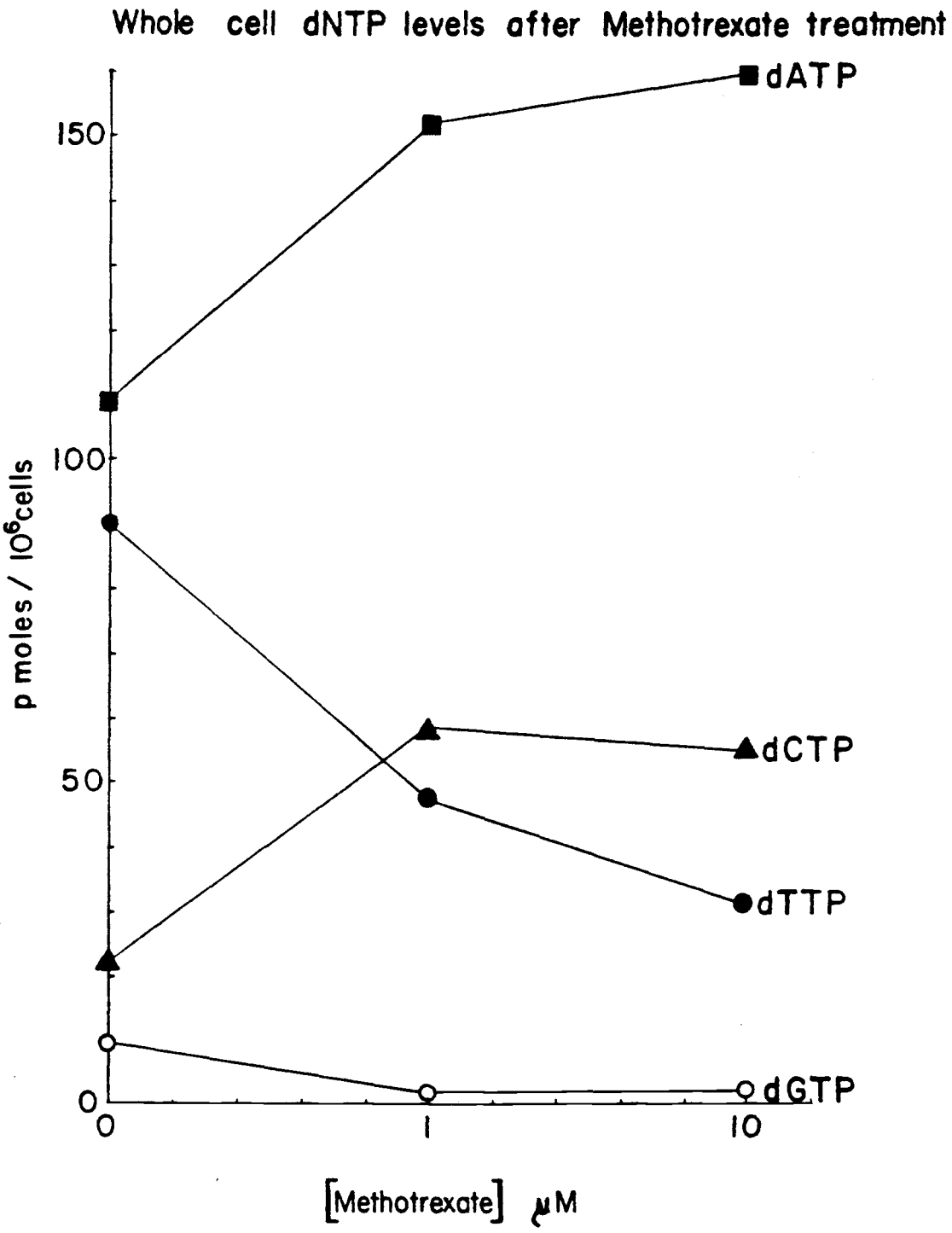

Figure 20. Changes in the levels of HeLa cell dNTPs as a function of methotrexate concentration.

and dCTP are increased over control levels and dGTP is dropped to very low levels at both methotrexate concentrations.

Comparison of the methotrexate effects on mitochondria and whole cells illustrates several differences. First, dATP pools are increased in both, but mitochondria have a fractionally higher increase. Second, whole cell dTTP pools are drastically reduced in whole cells but increased in mitochondria at ten micromolar and unchanged at one micromolar. This is the most significant result from these data since methotrexate is thought to exert its antimetabolic effects by lowering dTTP levels. Third, dGTP pools are not affected by methotrexate in mitochondria but in whole cells are much reduced. Fourth, dCTP pools are dramatically increased in mitochondria as they are in whole cells but as with dATP the effect is much greater in mitochondria. Methotrexate does not significantly lower any mitochondrial pool at the concentrations studied whereas in whole cells both dGTP and dTTP are lowered.

Selective expansion of mitochondrial NTP pools was not confined to the deoxy series, as shown in Figure 21, which shows accumulation of all four rNTP pools in methotrexate-treated mitochondria, even as cellular pools of all four nucleotides are declining. Fluorodeoxyuridine treatment has comparable effects on rNTP pools, as shown in Figure 22. Cellular rNTP pools all shrink, although not to the same extent as with methotrexate, but the most striking effect is the accumulation of all four nucleotides in mitochondria as a result of fluorodeoxyuridine treatment.

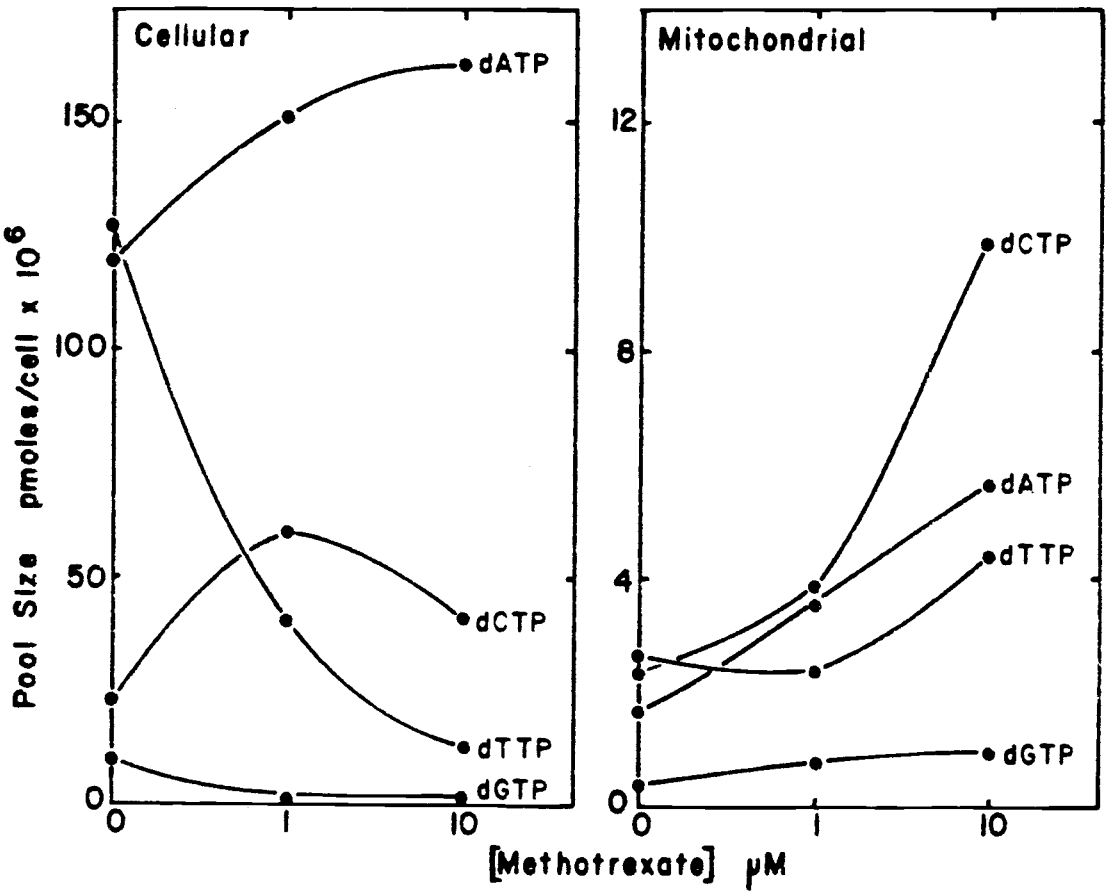

Figure 21. Effects of methotrexate on cellular and mitochondrial rNTP pools. The extracts from the experiments described in Figures 19 and 20 were analyzed for rNTP content by HPLC as described in Methods.

Figure 22. Effects of fluorodeoxyuridine on cellular and mitochondrial rNTP pools. Experimental conditions were as described in Figure 21 except that drug treatment involved one μM fluorodeoxyuridine. F denotes data from the drug-treated culture, which are compared with corresponding data from an untreated culture.

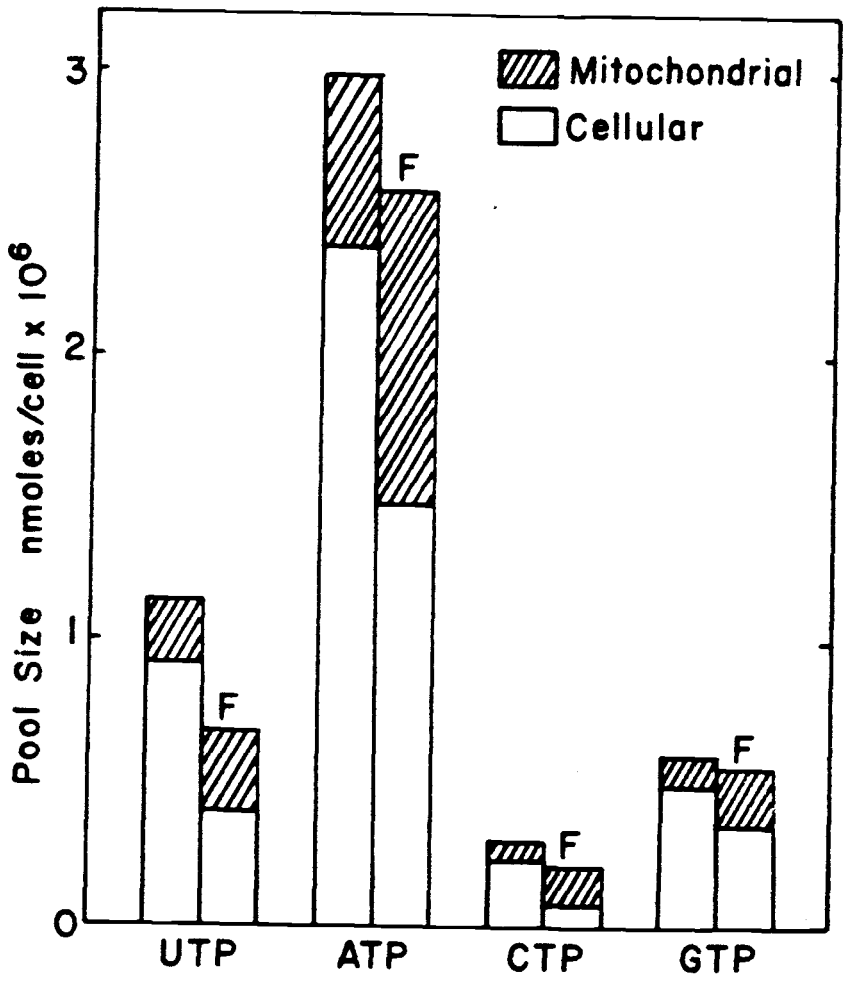

Figure 22. Effects of fluoro deoxyuridine on cellular and mitochondrial rNTP pools.

H. Deoxypyrimidine Supply in Mitochondrial and Nuclear DNA Synthesis In Mouse L-Cells

The rationale of these experiments was to illustrate the mechanism of synthesis of the proximal precursors to deoxypyrimidine nucleotides in the DNA of mouse L-cell mitochondria. When exogenous levels of uridine are high enough (200 μM), de novo synthesis of uridine is partially inhibited (100). When growing cells are supplied with [6-^{3}H] uridine, DNA can be labeled in both dCMP and dTMP positions (see Introduction and Fig. 1). By isolating nuclear and mitochondrial DNAs, digesting them to dNMPs and separating the deoxymononucleotides by high-performance liquid chromatography, one can determine the relative utilization of [^{3}H] uridine as dCTP and dTTP for DNA synthesis.

These experiments were performed by first prelabeling the cells with ^{32}P-orthophosphate for at least two cell doublings so that all pools of phosphorylated macromolecules should have reached close to steady state specific activities. This was followed by introduction of 200 μM [6-^{3}H] uridine and isolation of both mitochondrial and nuclear DNA at specific time intervals up to 96 hours. The medium was changed every 12 hours to prevent depletion of the exogenous uridine and ^{32}P-orthophosphate. At each time point the cells were harvested bytrypsinization, and nuclei and mitochondria isolated using the differential centrifugation method (see Methods). Nuclear DNA was purified and the nuclear DNA digested to dNMPs by using DNAse I and venom phosphodiesterase.

Isolation of mtDNA required further purification and was done by using a hybridization method. Shown previously in Figure 13

is HeLa cell mtDNA isolated from mitochondria prepared by the differential centrifugation method. Although there is a distinct mtDNA band at 15 kb, other cellular DNA contaminates these preparations. As a means of separating DNA a saturation hybridization approach was developed. Fifteen micrograms of cloned mouse L-cell mtDNA (obtained from D. A. Clayton) was cleaved with EcoRI, denatured and bound to nitrocellulose filters. The in vivo labeled mtDNA was also cleaved with EcoRI, denatured and hybridized to the nitrocellulose bound mtDNA. Hybridized DNA was removed from the filter by heating to 100°C, digested to dNMPs and separated by HPLC as described in Methods. By this method one can isolate 10^4 cpm of ^{32}P labeled mtDNA from two 175 cm^2 flasks.

In order to check the validity of this approach several experiments were done. First, shown in Figure 23 are the HPLC elution profiles for nuclear and mitochondrial DNA hydrolysates from mouse L-cells labeled with ^{32}P-orthophosphate for 72 hours and with 200 micromolar [5-^3H]-uridine for the last 24 hours. For both profiles the distribution of ^{32}P radioactivity corresponds to the published values for mouse nuclear and mitochondrial DNA nucleotide composition (22,114). The percent total recovered ^{32}P for each of these hydrolysates along with the published values for nucleotide composition are presented in Table V. The results of this experiment illustrate that exogenously supplied ^{32}P-orthophosphate labeled the DNAs to equivalent specific activities and that the ^{32}P radioactivity can be used as an indicator of mass.

Secondly, the design of the experiment shown in Figure 23 allows demonstration of another important control; no ^3H radioactivity

Table V. PERCENT RECOVERED ^{32}P RADIOACTIVITY IN NUCLEAR AND mtDNA HYDROLYSATES

Nucleotide	% ^{32}P Recovered		Published % Nucleotide Composition	
	mtDNA	Nuclear DNA	mtDNA (22)	Nuclear DNA (114)
dAMP	30.1	29.4	31.6	28.2
dTMP	29.3	29.9	31.6	29.1
dGMP	20.9	20.5	18.3	21.0
dCMP	19.8	20.2	18.3	21.1

Figure 23. HPLC elution profile of dNMPs derived from nuclear and mtDNAs labeled with [5-^{3}H]-uridine. Cells were prelabeled for 48 hours with ^{32}P and labeled with 200 μM [5-^{3}H]-uridine for an additional 24 hours in the continuing presence of ^{32}P. Cells were harvested and DNAs were purified, enzymatically digested to 5'-dNMPs, and separated by HPLC as described in Methods. Each fraction represents one milliliter of eluate.

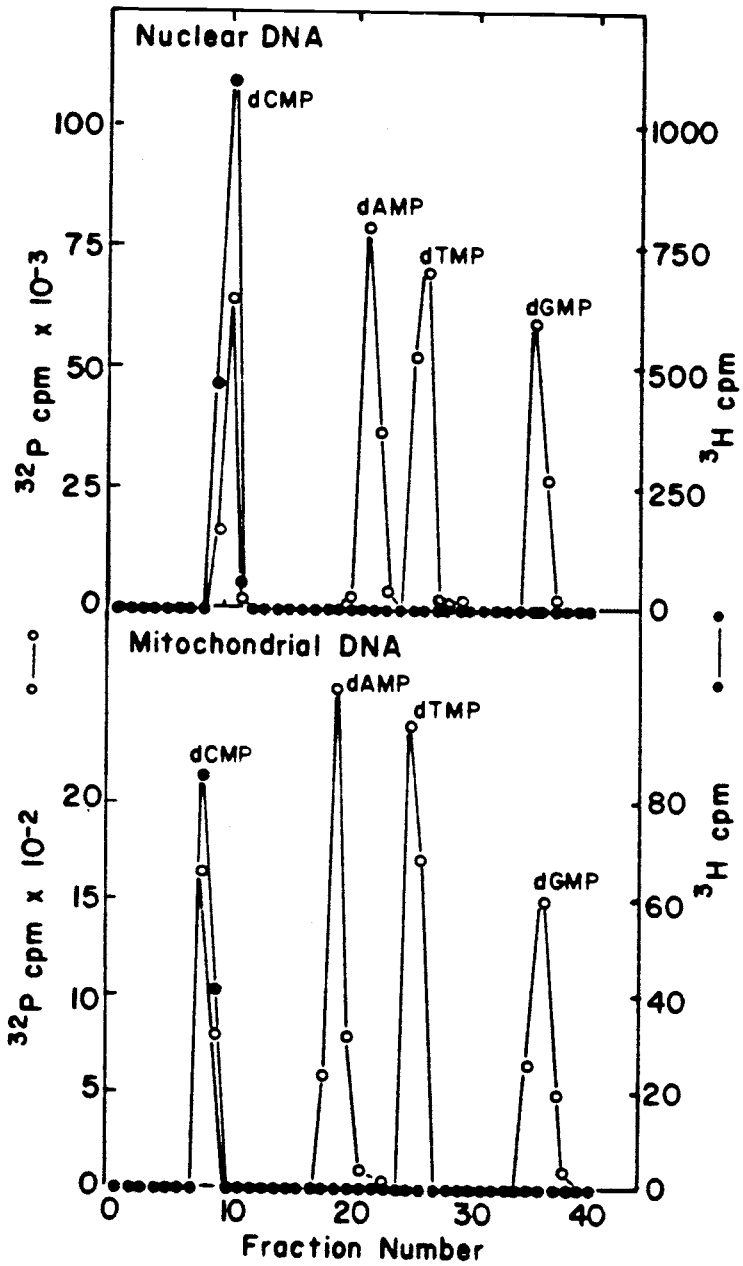

Figure 23. HPLC elution profile of dNMPs derived from nuclear and mtDNAs labeled with [5-^{3}H]-uridine.

is detectable in either dTMP fraction. Because the cells were labeled with [5-^{3}H]-uridine, dTMP residues should not be labeled. The reason for this is the thymidylate synthetase reaction displaces the hydrogen atom (a tritium atom in this experiment) at the 5 position on the pyrimidine ring. Because no radioctivity is seen in the dTMP peak this demonstrates the absence in these DNA hydrolysates of any contaminating RNA hydrolysis products. UMP residues would still be radioactive and would cochromatograph, if present, with dTMP under the HPLC elution conditions employed.

Since the ^{32}P radioactivity present in each nucleoside monophosphate is at a steady state specific activity and is representative of the nucleotide's mass, it is possible to represent tritium specific activity by the ^{3}H/^{32}P ratio. Shown in Figure 24 are the ^{3}H/^{32}P ratios for HPLC purified dCMP residues from mtDNA and nuclear DNA. The value for the mitochondrial dCMP ratio is nearly twice that of the nuclear dCMP ratio and this difference is shown to continue for up to three days. These values are believed to correspond to the steady state specific activities of the proximal dCTP pools used in the replication of the two different DNAs.

In order to extend to dTMP the observation described for mtDNA residues, [6-^{3}H]-uridine was used as the deoxypyrimidine precursor. Position six on the pyrimidine ring is unmodified in both dTTP and dCTP biosynthesis; therefore, dCMP and dTMP residues in nuclear and mtDNA are labeled when exogenous [6-^{3}H]-uridine is supplied to growing cells. Shown in Figures 25 and 26 are the ^{3}H/^{32}P ratios in nuclear and mtDNA dCMP and dTMP residues, respectively. Again, the selectively greater utilization of exogenously supplied

Figure 24. Utilization of exogenously supplied [5-^{3}H]-uridine for synthesis of dCMP residues derived from nuclear and mtDNA. The labeling conditions are identical to those described in Figure 23. Shown are the $^{3}H/^{32}P$ ratios at 1, 2, and 3 days for the dCMP peaks obtained by HPLC. Mitochondrial values have an approximate ten percent error due to low levels of ^{3}H.

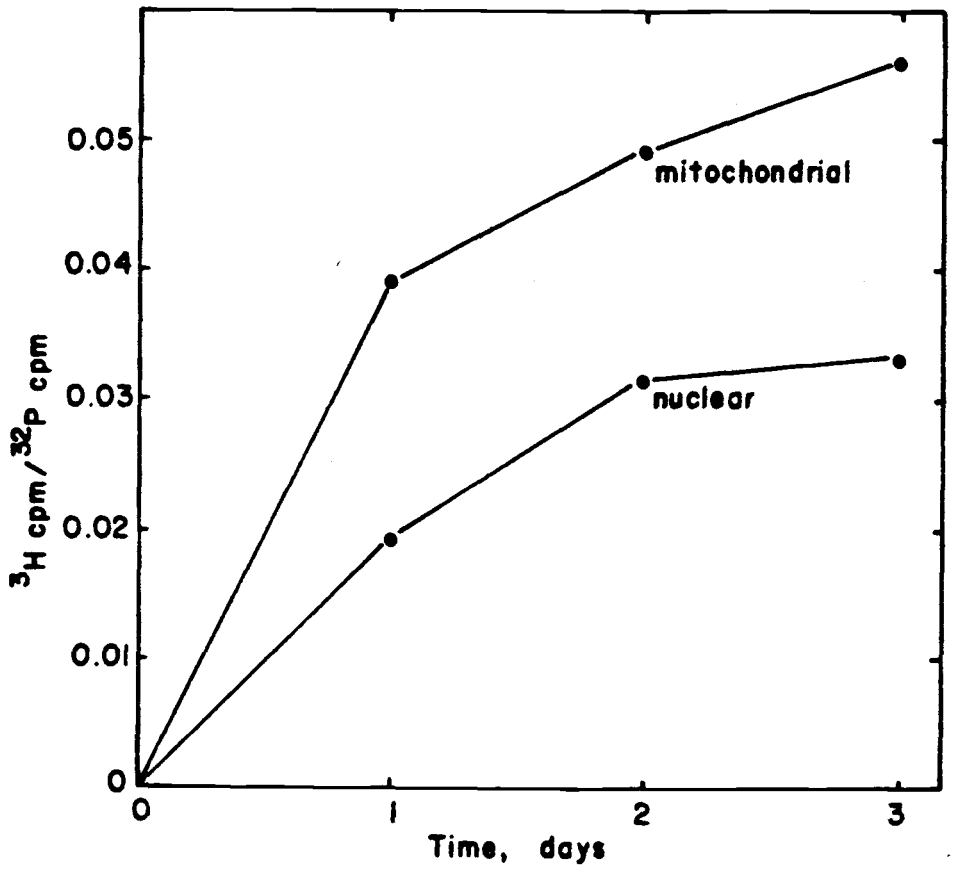

Figure 24. Utilization of exogenously supplied [5-^{3}H]-uridine for synthesis of dCMP residues derived from nuclear and mtDNA.

Figures 25 and 26. Utilization of exogenous [6-^{3}H]-uridine for DNA synthesis by mitochondria and nuclei in mouse L-cells. Cells were prelabeled for at least 48 hours with ^{32}P and starting at time zero with [6-^{3}H]-uridine in the continuing presence of ^{32}P. At the indicated time points nuclei and mitochondrial DNA were prepared, digested to dNMPs which were separated by high performance liquid chromatography and individual dNMPs counted for both ^{3}H and ^{32}P as described in Methods. The ^{3}H/^{32}P ratios (after subtraction of ^{32}P spill-over into ^{3}H) are given here. Mitochondrial dCMP and dTMP, ● ; nuclear dCMP and dTMP, ■ . Figure 25 is for dCMP and Figure 26 is for dTMP. Mitochondrial values have an approximate ten percent error due to low levels of ^{3}H.

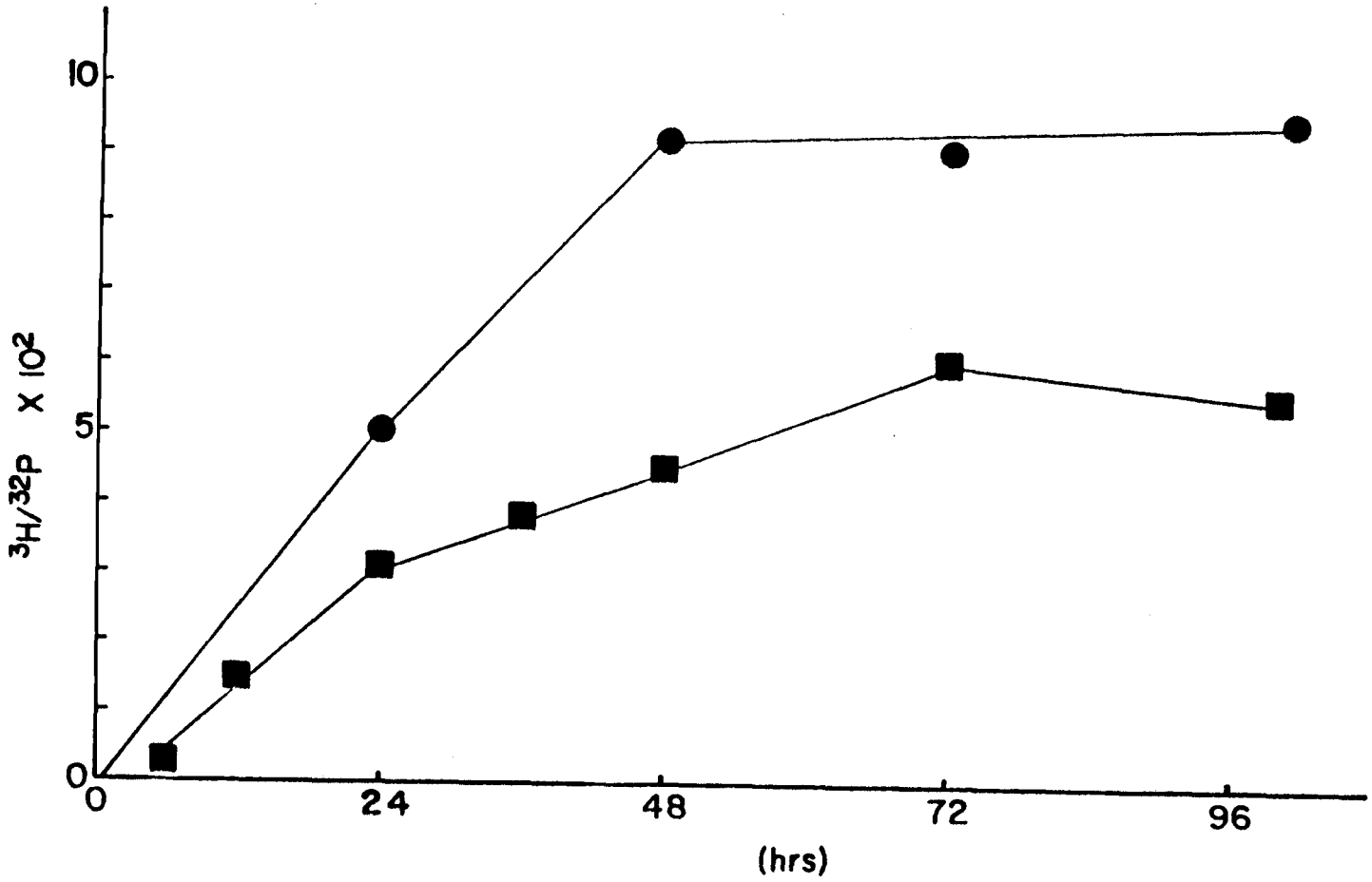

Figure 25. Utilization of exogenous [6-^{3}H]-uridine for DNA synthesis by mitochondria and nuclei in mouse L-cells.

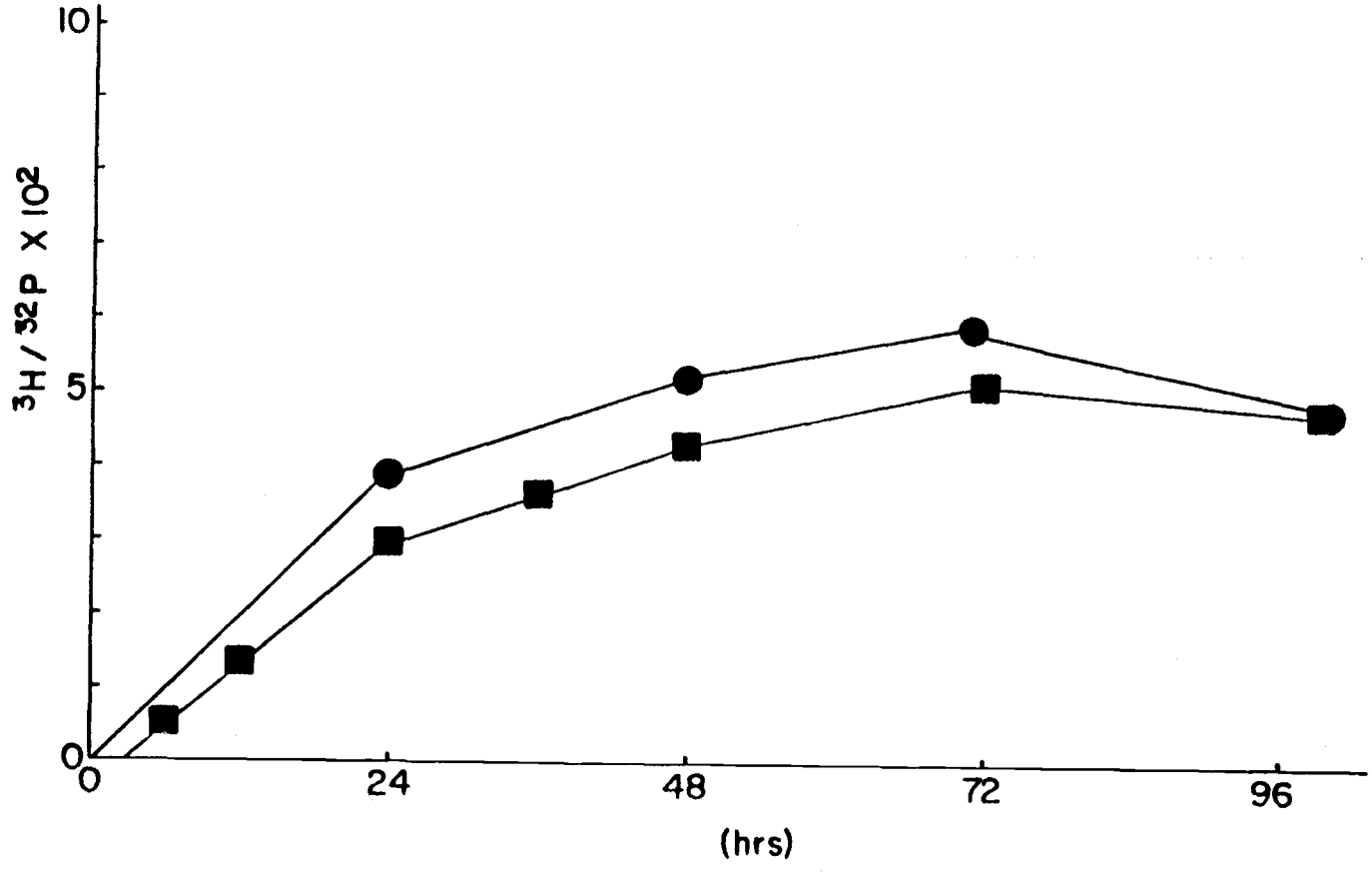

Figure 26. Utilization of exogenous [6-^{3}H]-uridine for DNA synthesis by mitochondria and nuclei in mouse L-cells.

uridine for mitochondrial dCMP residues is observed (Fig. 25). In contrast dTMP residues derived from mtDNA and nuclear DNA are labeled to equivalent specific activities (Fig. 26).

In these experiments if DNA-derived deoxypyrimidine monophosphates were synthesized entirely from exogenous uridine, then an expected steady state $^{3}H/^{32}P$ ratio can be calculated by using specific activities and counting efficiencies of the ^{32}P and ^{3}H-uridine. This calculation was made and the expected ratio is 0.087, which is very close to the values observed for dCMP residues in mtDNA (Fig. 25). This implies that mouse L-cells when presented with 200 micromolar exogenous uridine synthesize all of the dCTP pool for mtDNA synthesis from this exogenous source of pyrimidines. In contrast dTTP pools for nuclear and mtDNA synthesis and dCTP pools for nuclear DNA synthesis are derived partially from exogenous uridine and partially from other sources, presumably de novo synthesis, of pyrimidines that are not labeled with exogenous uridine (Figs. 25 and 26).

IV. DISCUSSION

The work presented in this dissertation has provided evidence that mammalian mitochondria contain independently regulated pathways for the biosynthesis of DNA precursors. This proposal is supported by data obtained from two novel experimental approaches. First is the direct quantitation of intramitochondrial dNTP levels and how they vary in response to antimetabolic stress. The second approach analyzes the radioactive labeling of DNA with an exogenously supplied deoxypyrimidine precursor. The following discussion focuses on how nuclear and mtDNA precursor biosynthesis differ.

A. Quantitation of HeLa Cell Mitochondrial dNTPs

The development of methodologies necessary to measure the small pools of dNTPs in HeLa cell mitochondria was aided by an enzymatic assay for dNTPs sensitive enough to measure one picomole of an individual dNTP, the relative ease of growing large numbers of HeLa cells in suspension culture, and a membrane solubilization agent that leaves mitochondria intact. It is significant that these mitochondrial pools are large enough to quantitate and the values obtained from growing cells seem to fall into a range of values that one might expect a priori. These expected values are derived from two considerations. First, HeLa cell mitochondria contain about one percent as much DNA as the nucleus; therefore, the mitochondrial dNTP pools might have been expected to be about one percent of the cellular dNTP pools, reflecting the smaller genome size. Secondly, the dNTP pools might have been expected to reflect the volume of HeLa cell mitochondria with respect to total cell volume which has been reported to be seven percent (92) using serological methods (116). The

observed values range close to both of the expected values; dATP, 1.4%; dTTP, 3.0%; dGTP, 10.0%; dCTP,10.4%.

Mammalian mitochondria are known to replicate their DNA throughout the cell cycle (36) whereas nuclear replication occurs only during the S-phase. Concomitant to nuclear DNA replication is a reported increase in nuclear dNTP levels (117). The percent of cellular dNTP pool values for mitochondria given above are representative of cells distributed throughout the cell cycle. If these same measurements were made using cells synchronized in S-phase it is very possible these values would decrease as the cellular dNTP pools increase.

The values for mitochondrial dNTP pools are corrected to reflect the mtDNA yield in the various experiments. This was necessitated by the incomplete recovery of mitochondria during the rapid subcellular fractionation procedure. Therefore the mitochondrial pools are calculated on a per mass of mtDNA basis. This rationale is supported by the reports of Posakony et al. (118), who showed that HeLa cells maintained a constant mitochondrial volume during all stages of the cell cycle and Berk and Clayton (26), who demonstrated a constant number of mtDNA genomes per mouse L-cell. Furthermore, replication of mtDNA occurs throughout the cell cycle (36). The assumption that mtDNA is a good indicator of mitochondrial volume seems valid.

Another consideration crucial to the validity of these results is the effectiveness of the cell lysis methodology which utilizes digitonin. Digitonin lyses cells by complexing with β-hydroxysterols which are present at a higher concentration in the

plasma membrane relative to the inner mitochondrial membrane. Digitonin is also known to solubilize the outer mitochondrial membrane presumably because of a higher β-hydroxysterol content. Because others have shown the presence of "nonspecific diffusion channels" in outer mitochondrial membranes (119) maintenance of this membrane seem unnecessary especially when quantitation of inner mitochondrial matrix metabolites is the objective. Furthermore, the location and replication of mtDNA is within the inner mitochondrial matrix (45). Because of these considerations it seems reasonable to expect the compartment containing dNTPs for mitochondrial replication to be maintained intact during cell lysis with digitonin.

Often it is desirable to express metabolite levels as molar concentrations. The calculation of mitochondrial nucleotide pools as molar concentrations would have been preferred in these studies as well. Unfortunately the accepted structure of the inner mitochondrial matrix makes such calculations highly dependent on assumptions about the hydration state of the matrix. Determinations of the matrix protein concentration range from 200 to 560 µg/ml depending on the metabolic state of the mitochondria (120,121). Under these conditions protein molecules are very close to each other and the amount of water not bound to proteins is very small. Values for the hydration state of matrix proteins are at best theoretical estimates (120). Matrix protein concentrations vary according to metabolic state and probably mitochondrial isolation methods. These two considerations place a large degree of error into any matrix free water value, a number essential for determination of mitochondrial nucleotide concentration.

For these reasons nucleotide pools in this thesis are presented on a mass per cell basis.

B. Antimetabolite Effects on Cellular Nucleoside Triphosphate Pools

The observation that mitochondrial dNTP pools are selectively resistant to depletion by either methotrexate or fluorodeoxyuridine is the most significant generalized result of these experiments. This result corroborates the observations of Bogenhagen and Clayton who demonstrated the relative resistance of mtDNA replication to methotrexate and fluorodeoxyuridine (91). The results in this dissertation do not support their suggestion that mtDNA replication is resistant to antimetabolite induced dTTP depletion but rather lend support to the idea that mitochondria represent a metabolic compartment relatively resistant to DNA precursor depletion. Any explanation for mitochondrial dNTP retention and continued mtDNA replication in the presence of these antimetabolites must take into consideration the simultaneous accumulation of ribonucleoside triphosphates. One explanation that takes this consideration into account is that mitochondria are capable of synthesizing nucleoside triphosphates and that methotrexate and fluorodeoxyuridine are simply not transported into the inner mitochondrial matrix. Because thymidine is readily taken up by mitochondria and incorporated into mtDNA (91) it seems likely the analogous fluorodeoxyuridine would also be taken up by mitochondria. Methotrexate might not be transported into mitochondria. The use of fluorescent methotrexate analogs could yield information concerning mitochondrial methotrexate transport.

As stated previously, the inner mitochondrial matrix represents a unique biochemical environment (120). Because of this

the possibility that methotrexate and fluorodeoxyuridine do not function as they would in a more aqueous environment must be given serious consideration as an explanation for the selective maintenance of DNA precursors in mitochondria.

The effects of methotrexate and fluorodeoxyuridine on whole cell dNTP levels were mostly anticipated. Thymidine triphosphate levels decreased because of the inhibition of thymidylate synthesis. Variations in the other dNTP levels can be rationalized in terms of the dTTP decrease and the reported allosteric regulations of mammalian ribonucleotide diphosphate reductase (7). The decrease in dGTP levels is probably the result of a lower activity of GDP reduction due to dTTP being the prime positive effector for reduction of GDP to dGDP. The increase in dATP levels can also be explained in terms of ribonucleotide reductase regulation since dTTP is a negative effector of ADP reduction. Elford et al. (122) have shown the activity of CDP reduction in HeLa cells increases after treatment with either methotrexate or fluorodeoxyuridine. This could explain the observed increase in dCTP levels.

C. Compartmentation of Precursors for Nuclear and Mitochondrial DNA in Mouse L-Cells

The design of this line of experimentation was to determine whether novel mechanisms for nuclear and mtDNA precursor synthesis existed. The experimental results clearly indicate the existence of two distinctly regulated pools of dCTP in mouse L-cells. This is best demonstrated in Figures 25 and 26. Under these conditions synthesis of mitochondrial dCTP is drawn entirely from exogenous uridine. In contrast is mitochondrial dTTP which is derived only partially from

the exogenous uridine source and hence resembles nuclear dCTP and dTTP synthesis. The mitochondrial dTTP result does not corroborate the observations of Bogenhagen and Clayton (91) or the conclusions presented elsewhere in this dissertation, which indicate mitochondrial dTTP pools are compartmentalized. Presumably this is because the mitochondrial mechanism for dTTP synthesis resembles the nuclear mechanism in terms of utilizing uridine as a precursor.

As shown in Figure 1, the entrance of uridine into the metabolic pathway for deoxypyrimidine biosynthesis is at a point quite distal to DNA synthesis. An explanation for the compartmentation observed in these experiments is possible using the observations of Wiegers et al. (100), whose methods were used as a basis for the experiments presented here. They demonstrated that HeLa cells used different precursor pools for the synthesis of ribosomal and messenger RNAs. Interestingly, their data for RNA resemble closely my data for mtDNA. In their hands addition of 200 μM [5-^{3}H]-uridine to HeLa cell cultures resulted in mRNA being labeled to the full specific activity of the exogenous [S-^{3}H]uridine while rRNA was labeled to about half this level. This difference was observed for both CMP and UMP in both RNAs. Their results strongly suggest the existence of compartmentalized pools of CTP and UTP for RNA synthesis. For uridine to be incorporated into DNA it must pass through UTP and then be converted to CTP by CTP synthetase (see Figure 1). I propose to explain the results in Figures 25 and 26 by suggesting mitochondrial dCTP is drawn from the full specific activity pools of CTP and UTP destined for mRNA whereas nuclear dCTP is drawn from the CTP and UTP pools destined for rRNA synthesis. In contrast I propose dTTP, which has been shown to

be derived primarily from dCMP via dCMP deaminase (61), is drawn from the UTP and CTP pools destined for rRNA. This model incorporates the data of Wiegers et al. as well as the data presented in this dissertation into a model explaining cellular pyrimidine biosynthesis in general.

A key to understanding how nucleic acid precursors can be channeled depending on their ultimate fate lies in the proposal that enzymes of nucleotide biosynthesis interact to form multienzyme complexes. These complexes are believed to pass intermediates directly from one enzyme to another to create a functional compartmentalization. Multienzyme complexes for deoxynucleotide biosynthesis were first demonstrated in prokaryotes (123,124,125) and later the possibility of their existence in eukaryotes (126). Whether or not such complexes are actually involved in the phenomena described in this dissertation was not addressed experimentally but the possibility is intriguing and merits further experimentation.

The two experimental approaches described in this thesis combine to shed some light on nucleotide biosynthesis in mammalian cells. It is apparent separate sites of deoxyribonucleotide biosynthesis exist and that cells contain independently regulated pathways to dNTP synthesis for nuclear and mtDNA. Much has yet to be learned about the role of nucleotide transport within the cell, fluctuation of nucleotides during the cell cycle, effects of exogenous DNA precursors on nucleotide concentrations, and the role of multienzyme complexes in the biosynthesis of nucleotides. The techniques and results in this thesis should contribute something to those pursuing a deeper understanding of these processes.

V. REFERENCES

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