

DNA SYNTHESIS IN A PURINE  
AUXOTROPHIC MUTANT OF CHINESE HAMSTER CELLS

BY



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A thesis submitted to the Faculty of Graduate Studies  
and Research of McGill University in partial fulfillment  
of the requirements for the degree of Doctor of Philosophy.

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Montreal

August 1979

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Ph. D. Thesis

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## ABSTRACT

When V79 pur 1 cells are maintained in the absence of purines, the cells stop growing but remain viable for up to 48 h. Within the first 6 h after purine deprivation DNA synthesis is inhibited and the cells are arrested in S phase. The purine deoxyribonucleoside triphosphate (dNTP) pools decrease in size and the pyrimidine (dNTP) pools expand. Restoration of purine nucleotides into the medium results in normal rates of DNA synthesis both in vivo and in vitro. Kinetic analyses of DNA synthesis in samples pulsed for 1 min and chased for 1 h show a delay in maturation of newly replicated Okazaki-type pieces to full size DNA. DNA fiber autoradiography reveals a retardation in the rate of replication fork movement but no apparent inhibition of initiation in active clusters of replication units. The results indicate that purine deprivation upsets the intracellular dNTP pool balance and inhibits the elongation of nascent DNA chains.

SYNTHÈSE DE L'ADN DANS UN MUTANT DE HAMSTER CHINOIS  
AUXOTROPHE DES PURINES

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Thèse de Doctorat

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RÉSUMÉ

Quand les cellules V79 pur 1 sont maintenues dans un milieu sans purines, elles arrêtent de pousser mais restent vivantes jusqu'à 48 h. Dans les premières 6 h qui suivent la privation des purines la synthèse de l'ADN est inhibée et les cellules sont arrêtées dans la phase S du cycle cellulaire. La grandeur des 'pools' des déoxynucleosides triphosphates (dNTP) des purines diminue et celle des pyrimidines augmente. Le taux normal de la synthèse d'ADN résulte de la restauration des nucléotides des purines dans le milieu aussi bien in vivo qu'in vitro. Une analyse cinétique de la synthèse de l'ADN montre un délai dans la maturation des fragments du type Okazaki nouvellement synthétisés en longues chaînes d'ADN. L'autoradiographie des fibres d'ADN révèle un retardement du mouvement de la fourche de réplication. Par contre cette même technique ne met pas en évidence une inhibition d'initiation dans les groupes d'unités de replication qui sont actifs. Les résultats indiquent que la privation des purines renverse la balance des 'pools' des dNTPs cellulaires et inhibe l'élongation des chaînes d'ADN nouvellement synthétisées.

## TABLE OF CONTENTS

ABSTRACT (English)	
RÉSUMÉ (French)	
TABLE OF CONTENTS	
LIST OF FIGURES	i
LIST OF TABLES	iii
ACKNOWLEDGEMENTS	v
PREFACE	vi
CHAPTER 1 - GENERAL INTRODUCTION	1
I. DESCRIPTION OF THE MUTANT	2
II. NUCLEOTIDE POOLS IN ANIMAL CELLS	3
III. NUCLEOTIDE METABOLISM	5
A. PURINE NUCLEOTIDE METABOLISM	6
a) Purine Nucleotide Biosynthesis de novo	6
b) Purine Nucleotide Synthesis from Bases and Nucleosides	8
c) Interconversion of Purine Nucleotides	9
B. PYRIMIDINE NUCLEOTIDE METABOLISM	11
a) Pyrimidine Nucleotide Biosynthesis de novo	11
b) Pyrimidine Nucleotide Synthesis from Bases and Nucleosides	13
c) Interconversion of Pyrimidine Nucleotides	14
IV. THE CELL CYCLE	14
The temporal Structure of S Phase	18
V. DNA SYNTHESIS	20
a) Units of Replication	20
b) Initiation of DNA Replication	21
c) Clustering of Replication Units	22

d) Discontinuous DNA Synthesis	23
e) In vitro DNA Synthesis	26
f) Enzymes involved in DNA Replication	27
VI. HISTONE SYNTHESIS AND THE S PHASE	29
VII. OVERALL SUMMARY	31
VIII. REFERENCES	34

CHAPTER 2 - EFFECT OF PURINE DEPRIVATION ON DNA SYNTHESIS AND DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS OF A MAMMALIAN PURINE AUXOTROPHIC MUTANT CELL LINE.	62
Abstract	63
Introduction	64
Experimental Procedures	65
Results	68
Discussion	74
References	79
Tables	83
Figures	85
Connecting Text	90

CHAPTER 3 - INHIBITION OF DNA CHAIN ELONGATION IN A PURINE AUXOTROPHIC MUTANT OF CHINESE HAMSTER.	91
Abstract	92
Introduction	93
Experimental Procedures	94
Results	97

Discussion	105
References	109
Tables	115
Figures	119
CHAPTER 4 - GENERAL DISCUSSION	125
Introduction	
Limitations of the use of <sup>3</sup> H-thymidine as a monitor of DNA synthesis	125
Comparison between <sup>3</sup> H-thymidine incorporation into acid insoluble material and into S phase cells	127
Limitations of the use of Fluoromicrofluorography as a monitor of S phase	129
Analysis of the deoxyribonucleoside triphosphate pools	131
In vitro DNA synthesis	134
Histone synthesis and DNA synthesis	136
Experimental drawbacks regarding the characterization of the newly synthesized DNA	138
References	141
Table	145
Figure	146
OVERALL CONCLUSION AND PERSPECTIVES	147
APPENDIX	149
CLAIM TO ORIGINAL RESEARCH	155

LIST OF FIGURESCHAPTER 1

- FIGURE 1 - The Four-Factor Model for Nucleotide Pools.
- FIGURE 2 - Model for the in Vivo Regulation of Ribonucleotide Reductase Activity.
- FIGURE 3 - General Scheme for the Biosynthesis of Purine Bases and Nucleotides.
- FIGURE 4 - Purine Nucleotide Biosynthesis de Novo.
- FIGURE 5 - Purine Nucleotide Synthesis from Bases and Nucleosides.
- FIGURE 6 - Interconversion of Purine Nucleotides.
- FIGURE 7 - General Scheme for the Biosynthesis of Pyrimidine Bases and Nucleotides.
- FIGURE 8 - Pyrimidine Nucleotide Biosynthesis de Novo.
- FIGURE 9 - Pyrimidine Nucleotide Synthesis from Bases and Nucleosides.
- FIGURE 10- Normal Routes of Pyrimidine Nucleoside Triphosphate Formation in Animal Cells.

CHAPTER 2

- FIGURE 1 - Effect of Different Culture Media on the Growth of V79 pur 1 Cells.
- FIGURE 2 - Effect of Purine Deprivation on <sup>3</sup>H-thymidine Incorporation into Acid Insoluble Material.
- FIGURE 3 - Effect of Purine Deprivation on Cell Cycle Distribution of V79 pur 1 Cells.
- FIGURE 4 - Effect of Purine Deprivation on Deoxyribonucleoside Triphosphate (dNTP) Pools in V79 pur 1 Cells.

FIGURE 5 - In Vitro DNA Synthesis in Purine-Deprived V79 pur 1 Cells.

### CHAPTER 3

FIGURE 1 - CsCl Isopycnic Centrifugation of DNA Synthesized by Purine-Deprived and Purine-Supplemented V79 pur 1 Cells.

FIGURE 2 - Effect of Purine Deprivation on Histone Synthesis in V79 pur 1 Cells.

FIGURE 3 - Alkaline Sucrose Gradient Centrifugation of DNA Synthesized by Purine-Deprived V79 pur 1 Cells in Pulse-Chase Studies.

FIGURE 4 - Agarose Gel Electrophoresis of DNA Synthesized by Purine-Deprived V79 pur 1 Cells in Pulse-Chase Studies.

FIGURE 5 - DNA Fiber Autoradiograms from V79 pur 1 Cells.

FIGURE 6 - Cumulative Frequency Distributions of Intertrack Distances in Purine-Deprived and Purine-Supplemented V79 pur 1 Cells.

### CHAPTER 4

FIGURE 1 - Summary diagram.

### APPENDIX

FIGURE 1 - Alkaline Sucrose Gradient Centrifugation of DNA synthesized by V79 pur 1 Cells Deprived of Purines for 23 h.



LIST OF TABLESCHAPTER 1

TABLE 1 - Enzymes of Purine Ribonucleotide Biosynthesis de Novo.

TABLE 2 - Enzymes of Purine Ribonucleotide Synthesis from Bases and Ribonucleotides.

TABLE 3 - Enzymes of Purine Ribonucleotide Interconversion.

TABLE 4 - Enzymes of Pyrimidine Ribonucleotide Biosynthesis de Novo.

TABLE 5 - Enzymes of Pyrimidine Ribonucleotide Synthesis from Bases and Ribonucleosides.

CHAPTER 2

TABLE 1 - S Phase Cells in V79 pur 1 Following Purine Depletion.

TABLE 2 - Effect of Purine Deprivation on the Fraction of V79 pur 1 Cells Synthesizing DNA in vitro.

CHAPTER 3

TABLE 1 - Histone and DNA Synthesis in Purine-deprived and Purine-Supplemented (control) V79 pur 1 Cells.

TABLE 2 - Replication Fork Movement in Purine-Deprived and Purine-Supplemented V79 pur 1 Cells.

TABLE 3 - Replication Unit Lengths for Purine-Deprived and Purine-Supplemented V79 pur 1 Cells.

TABLE 4 - Frequency of Initiation in Purine-Deprived and Purine-Supplemented V79 pur 1 Cells.

CHAPTER 4

TABLE 1 - Ratios of Pyrimidine to Purine dNTP Pools in Purine-Depleted (D) and Control (C) V79 pur 1 Cells.

APPENDIX

TABLE 1 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material in V79 pur 1 Cells Depleted of Purines for 5, 6 or 7 h and then Supplemented with Hypoxanthine ( $30\ \mu\text{M}$ ; Repleted) for 1 h.

TABLE 2 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material in Purine-Depleted V79 pur 1 Cells.

TABLE 3 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material in Purine-Depleted V79 (wild-type) Cells.

TABLE 4 - Deoxyribonucleoside Triphosphate (dNTP) Pool Size in V79 (wild-type) Cells.

ACKNOWLEDGEMENTS

I would like to thank my research director, Dr. Roger Hand, for his insight, enthusiasm, encouragement and guidance during my sojourn in his laboratory.

Grateful acknowledgement is also extended to Dr. E.A. Baumann for his assistance and supervision in the in vitro experiments presented in chapter 2 and for helpful discussions; Dr. M. Meuth for his critical appraisal of the work presented in Chapter 2; and to Dr. M.W. Taylor for kindly providing the V79 and V79 pur 1 cell lines, as well as for his helpful suggestions concerning the experimental design.

I also want to thank Dr. W. Yaphe, chairman of the Graduate Committee of the Department of Microbiology and Immunology for his continuous interest and consideration, as well as the rest of the academic and non-academic staff of the department for their support during the time I was a graduate student.

Finally, I am grateful to my husband for always standing by, most of all in times of need.

PREFACE

With the permission of the Department of Microbiology and Immunology, and as provided for by the regulations of the Faculty of Graduate Studies and Research of McGill University (Section 7\*), Chapters 2 and 3 of this thesis are presented in the form of manuscripts suitable for submission to *The Journal of Biological Chemistry and Cell*, respectively.

Dr. E.A. Baumann, who is second author in Chapter 2, has contributed half the data in Table II of that chapter and supervised the experiments that appear in Figure 5 and Table II.

Dr. R. Hand, a co-author in both Chapters 2 and 3, has supervised the entire work presented in this thesis.

The work presented in this thesis is organized into four chapters:

Chapter 1 contains a review of the literature that is relevant to the work presented in Chapters 2 and 3.

Chapter 2 contains an analysis of the precursor deoxynucleotide pools in the purine-depleted V79 pur 1 cells. It also contains an analysis of the effect of purine depletion on the distribution of cells throughout the cell cycle and on the overall DNA synthesis.

Chapter 3 focuses on DNA replication per se in the mutant cells. It contains an analysis of the kinetics of DNA synthesis as well as a direct examination of the changes occurring at the replication fork.

Chapter 4 contains a supplemental discussion of the data presented in Chapters 2 and 3 as well as a discussion of the technical limitations.

Data that were not included in Chapters 2 and 3 are presented in the Appendix.

\* The full text of Section 7 is cited below:

7. Manuscripts and Authorship

The Candidate has the option, subject to the approval of the Department, of including as part of the thesis the text of an original paper, or papers, suitable for submission to learned journals for publication. In this case the thesis must still conform to all other requirements explained in this document, and additional material (e.g. experimental data, details of equipment and experimental design) may need to be provided. In any case abstract, full introduction and conclusion must be included, and where more than one manuscript appears, connecting texts and common abstract introduction and conclusions are required. A mere collection of manuscripts is not acceptable; nor can reprints of published papers be accepted.

While the inclusion of manuscripts coauthored by the Candidate and others is not prohibited for a test period, the Candidate is warned to make an explicit statement on who contributed to such work and to what extent, and Supervisors and others will have to bear witness to the accuracy of such claims before the Oral Committee. It should also be noted that the task of the External Examiner is much more difficult in such cases.

6

CHAPTER 1

GENERAL INTRODUCTION

6

## I. DESCRIPTION OF THE MUTANT

V79 pur 1 is a purine auxotrophic mutant of a Chinese hamster lung cell line that was obtained by treatment with bromodeoxyuridine and black light (Chu et al, 1972). The functional defect in these cells has been characterized by Feldman and Taylor (1975), who found that the biochemical block is at the level of phosphoribosylpyrophosphate amidotransferase (E.C.2.4.2.14), the first enzyme of the de novo purine biosynthetic pathway. A number of other purine auxotrophs has been generated, defective in various enzymes of the biosynthetic pathway (Holmes et al, 1976; Oates and Patterson, 1977; Patterson, 1975). Although these mutants are primarily used to confirm genetically and biochemically the functions of the deficient enzymes in the mammalian purine pathways, they also prove to be useful tools in the study of nucleic acid synthesis regulation. Purines eventually give rise to purine nucleotides which are incorporated into nucleic acids. The effect of lack of purines on RNA synthesis has been studied by Sriram and Taylor (1977), who found that upon purine deprivation macromolecular synthesis declines rapidly in these cells and the purine ribonucleotide pool size decreases 5-fold. On continuous purine starvation RNA and DNA synthesis resumes to reach approximately 30% of the normal level between 12 to 24 h. This is accomplished by a rise in the intracellular ribonucleotide pool level. Utilizing mengovirus, which gives a productive infection in V79 pur 1 cells even under conditions of starvation, they showed that ribosomal RNA is preferentially degraded and provides the nucleotides for RNA synthesis.

The effect of lack of purines on the intracellular deoxyribonucleotide pool levels and on DNA replication per se has never been reported in the literature. Deoxyribonucleotides are the building blocks of DNA and

have also been implicated in the regulation of its synthesis. In the study that follows this somatic mutant of Chinese hamster, in which the precise biochemical defect is known, has been used to analyse the effect of the mutation on the size of the intracellular deoxyribonucleoside triphosphate pools as well as on the ability of the mutant cells to initiate DNA synthesis and successfully replicate their DNA.

## II. NUCLEOTIDE POOLS IN ANIMAL CELLS

Control of nucleic acid synthesis by precursor pool concentrations has been frequently proposed in the literature. In general, a pool of any particular metabolite or family of metabolites is the total amount of the compound which is fed by and available to a set of enzymatic pathways. A four-factor model has been proposed according to which there are four basic pathways by which components may enter or leave the pool (Hauschka, 1973). As shown in Fig. 1, two processes (anabolism and salvage) supply the pool, and two others (catabolism and utilization) drain it.

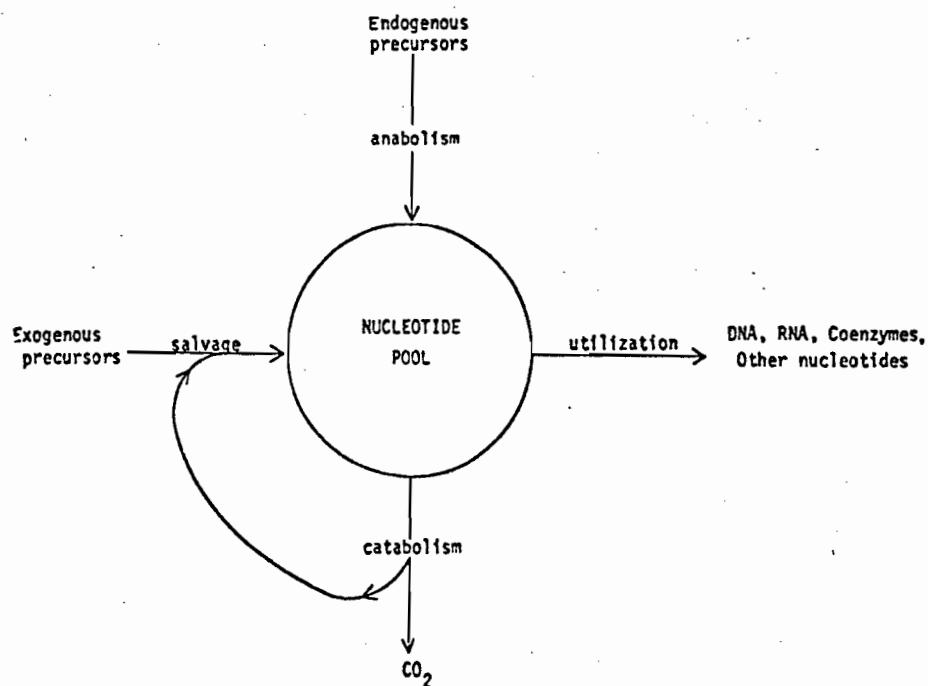


FIG. 1. The four-factor model for nucleotide pools; (from Hauschka, 1973).



5

Many studies have been concerned with the relationship between nucleotide pool sizes and the cell cycle. Ribonucleotide pools show little fluctuation in size whether in rapidly proliferating or density inhibited cell cultures (Colby and Edlin, 1970; Weber and Edlin, 1971). Deoxyribonucleotide pools on the other hand, show significant variations in size during the cell cycle (Nordenskjöld et al, 1970; Adams et al, 1971; Skoog and Nordenskjöld, 1971; Weber and Edlin, 1971; Bjursell et al, 1972). In general, the total deoxyribonucleotide pool in the cells amounts to about 1% of the ribonucleotide pool and it exists primarily in the triphosphate form (Gentry et al, 1965a; Cleaver, 1967). Resting cell populations contain small but definite amounts of all four triphosphates (Munch-Petersen et al, 1973; Nordenskjöld et al, 1970; Tyrsted, 1975; Walters et al, 1973). When cells in culture are stimulated to synthesize DNA, the pools increase in size before the onset of DNA synthesis and attain maximum levels during S-phase. Even though DNA synthesis requires an equal supply of all four bases, very large differences in pool size exist during S-phase; dGTP is always the smallest pool and dCTP usually the largest, the difference in some cases being up to 100-fold. Among the four pools the variations in dCTP most closely reflect the rate of DNA synthesis which has led to the suggestion that the dCTP pool might serve a regulatory role (Reichard, 1978). These findings were first established with cultured secondary mouse embryo cells (Nordenskjöld et al, 1970; Skoog and Nordenskjöld, 1971) and subsequently extended to many different cell lines (Soderhall et al, 1973; Bjursell et al, 1972; Skoog et al, 1973; Skoog et al, 1974; Walters et al, 1973).

6

Replication of DNA requires the continuous supply of building blocks, the four deoxynucleoside triphosphates, dATP, dGTP, dCTP and dTTP, that originate from the reduction of ribonucleotides. In *E. coli*

(Reichard, 1968) and in mammalian cells (Moore and Hurlbert, 1966; Reichard, 1968) reduction occurs at the disphosphate level and is catalysed by the enzyme ribonucleotide reductase (E.C.1.17.4.1) which is subject to allosteric regulation by the end-products, as shown in Fig. 2. Although it has been postulated (Reichard, 1978) that a product of ribonucleotide reductase, namely, one of the dNTPs, might serve a regulatory function in DNA replication, no strong evidence for such a relationship exists as yet.

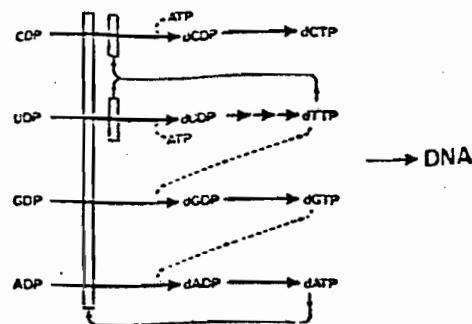


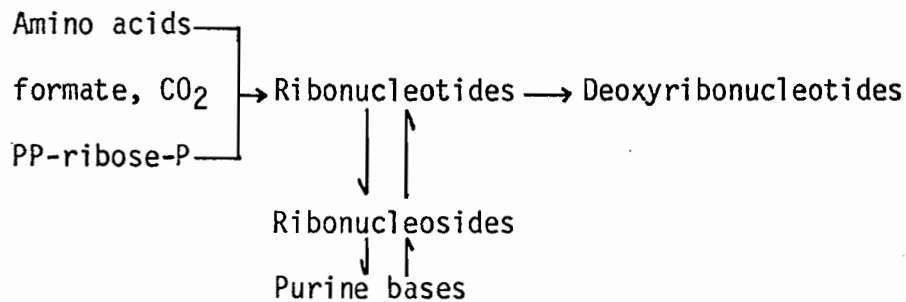
Figure 2. Model for the in vivo regulation of ribonucleotide reductase activity. Broken arrows indicate activations, bars indicate inhibitions. (from Reichard, 1978).

### III. NUCLEOTIDE METABOLISM

The biosynthesis of the ribonucleotides and deoxyribonucleotides is a central process for all cells, since the nucleotides are direct precursors of DNA and RNA and many also participate in metabolism as coenzymes. An important aspect of the biosynthesis of the nucleotides is the pathway of formation of their bases, the pyrimidines and purines. Nearly all living organisms, except for some bacteria, appear able to synthesize these bases from simpler precursors. The biosynthetic pathways leading to the nucleotides are under strict regulation.

### A. Purine Nucleotide Metabolism

Purine nucleotides may be synthesized de novo from amino acids and other small molecules or formed from preformed bases and ribonucleosides which may be derived from the diet or found in the environment of cells (Fig. 3).



#### a) Purine Nucleotide Biosynthesis de novo

In mammalian cells purine biosynthesis de novo serves for anabolic nucleotide synthesis. The pathway of purine nucleotide biosynthesis de novo is summarized below (Fig. 4). The enzymes involved are listed in Table 1.

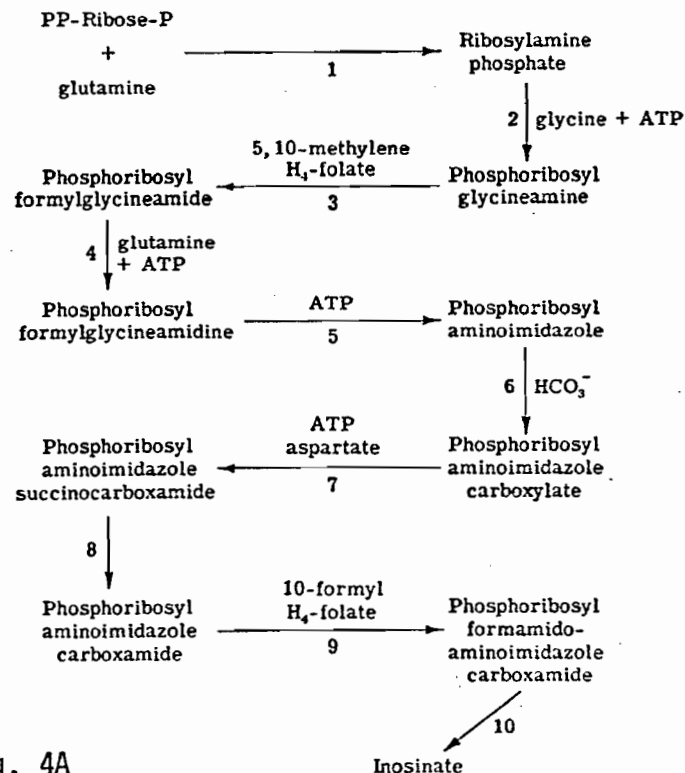


Fig. 4A

(from Henderson and Patterson, 1973)

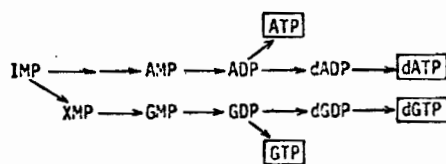


FIG.43. Normal routes of purine nucleoside triphosphate formation in animal cells. (from Hauschka, 1973).

TABLE 1  
ENZYMES OF PURINE RIBONUCLEOTIDE BIOSYNTHESIS *de Novo*<sup>a</sup>

Enzyme Commission number	Systematic name	Trivial name	
1	2.4.2.14	Ribosylamine-5-phosphate: pyrophosphate phosphoribosyltransferase (glutamate-amidating)	Phosphoribosyl pyrophosphate amidotransferase
2	6.3.1.3	Ribosylamine-5-phosphate: glycine ligase (ADP)	Phosphoribosyl glycineamide synthetase
3	2.1.2.2	5'-Phosphoribosyl-N-formylglycineamide: tetrahydrofolate 5,10-formyltransferase	Phosphoribosyl glycineamide formyltransferase
4	6.3.5.3	5'-Phosphoribosyl-formylglycineamide: L-glutamine amido-ligase (ADP)	Phosphoribosyl formylglycineamidine synthetase
5	6.3.3.1	5'-Phosphoribosyl-formylglycineamidine cyclo-ligase (ADP)	Phosphoribosyl aminoimidazole synthetase
6	4.1.1.21	5'-Phosphoribosyl-5-amino-4-imidazolecarboxylate carboxylase	Phosphoribosyl aminoimidazole carboxylase
7	6.3.2.6	5'-Phosphoribosyl-4-carboxy-5-aminoimidazole: L-aspartate ligase (ADP)	Phosphoribosyl aminoimidazole succinocarboxamide synthetase
8	4.3.2.2	Adenylosuccinate AMP-lyase	Adenylosuccinate lyase
9	2.1.2.3	5'-Phosphoribosyl-5-formamide-4-imidazolecarboxamide: tetrahydrofolate 10-formyltransferase	Phosphoribosyl aminoimidazole carboxamide formyltransferase
10	3.5.4.10	IMP 1,2-hydrolase (decyclizing)	IMP cyclohydrolase

<sup>a</sup> Numbers in the first column refer to reactions in the summary diagram (p. 6) (from Henderson and Patterson, 1973).

Purine biosynthesis de novo is regulated through the activity of the phosphoribosylpyrophosphate (PP-rib-P) amidotransferase, catalysing the first step of this pathway. The activity of the amidotransferase is regulated both by the availability of substrates, PP-rib-P and glutamine as well as by the concentration of the nucleotide end-products of the pathway, which exert feedback inhibition on this enzyme (Holmes et al, 1973; Wood and Seegmiller, 1973).

#### b) Purine Nucleotide Synthesis from Bases and Nucleosides

The requirement of cells for the de novo pathway is conditional due to the existence of purine salvage enzymes allowing cells to survive and grow in the presence of exogenous purine bases or nucleosides as their sole source of purines. These routes are summarized in Fig. 5. The enzymes are listed in Table 2.

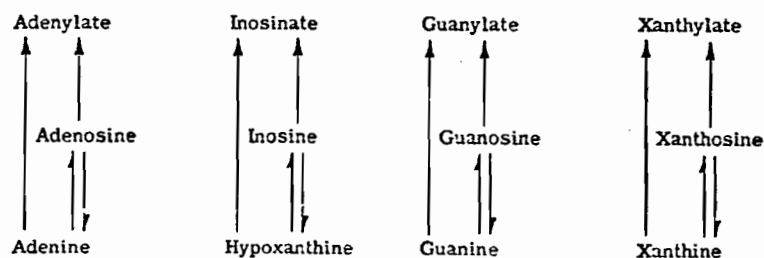


Fig. 5. Purine nucleotide synthesis from bases and nucleosides. (from Henderson and Patterson, 1973).

TABLE 2  
 ENZYMES OF PURINE RIBONUCLEOTIDE SYNTHESIS FROM BASES AND  
 RIBONUCLEOSIDES

Enzyme Commission number	Systematic name	Trivial name
2.4.2.7	AMP:pyrophosphate phosphoribosyltransferase	Adenine phospho- ribosyltransferase
2.4.2.8	IMP:pyrophosphate phospho- ribosyltransferase	Hypoxanthine- guanine phospho- ribosyltransferase
—	—	Xanthine phospho- ribosyltransferase
—	—	2,4-Dioxypyrimidine phosphoribosyl- transferase
2.7.1.20	ATP:adenosine 5'-phospho- transferase	Adenosine kinase
—	—	Inosine kinase
—	—	Guanosine kinase
2.4.2.1	Purine-nucleoside:orthophos- phate ribosyltransferase	Purine nucleoside phosphorylase
—	—	Nucleoside phospho- transferase

In mammalian tissue, purine nucleotide level is maintained, in addition to synthesis de novo, also by salvage synthesis from preformed purine bases. The latter synthesis is catalysed mainly by hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and associated with consumption of PP-rib-P.

### c) Interconversion of Purine Nucleotides

The pathways by which purine nucleotides are interconverted are summarized in Fig. 6. The enzymes are listed in Table 3.

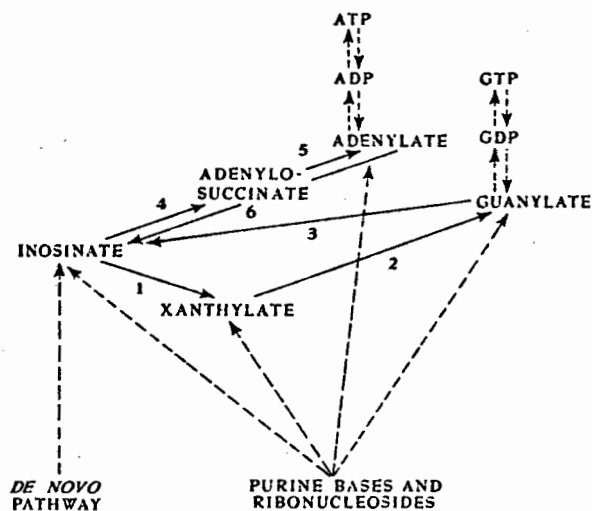


Fig. 6. Interconversion of purine nucleotides.  
(from Henderson and Patterson, 1973).

TABLE 3  
ENZYMES OF PURINE RIBONUCLEOTIDE INTERCONVERSION\*

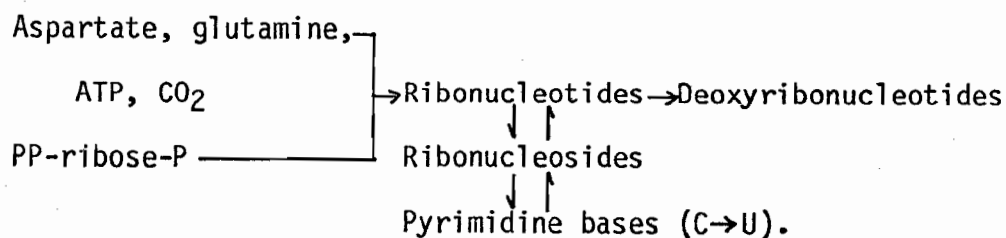
Enzyme Commission number	Systematic name	Trivial name
1 1.2.1.14	IMP:NAD oxidoreductase	IMP dehydrogenase
2 6.3.4.1	Xanthosine-5'-phosphate:ammonia ligase (AMP)	GMP synthetase
2 6.3.5.2	Xanthosine-5'-phosphate:L-glutamine amido-ligase (AMP)	GMP synthetase
3 1.6.6.8	Reduced-NADP:GMP oxidoreductase (deaminating)	GMP reductase
4 6.3.4.4	IMP:L-aspartate ligase (GDP)	Adenylosuccinate synthetase
5 4.3.2.2	Adenylosuccinate AMP-lyase	Adenylosuccinate lyase
6 3.5.4.6	AMP aminohydrolase	AMP deaminase

\* Numbers in the first column refer to numbers in the summary diagram (Fig. 6)  
(from Henderson and Patterson, 1973).

The ability of cells to incorporate free purines is dependent on 1) purine transport, 2) the availability of PP-rib-P, 3) activity of the pertinent purine phosphoribosyltransferase, and 4) loss of the purine by catabolic routes. Hypoxanthine and adenine are the more commonly used purine precursors because their conversion to nucleotides is generally more efficient than for other purines (Hauschka, 1973). Hypoxanthine enters the adenine and guanine pools in equivalent amounts (Brockman et al, 1970) and its incorporation is very efficient under conditions in which the endogenous purine supply is reduced (Hryniuk, 1972). In this condition, exogenous hypoxanthine satisfies all the purine requirements of cultured cells (Hakala, 1957).

### B. Pyrimidine Nucleotide Metabolism

Pyrimidine nucleotides, like those of purines, may be synthesized de novo from amino acids and other small molecules or from preformed pyrimidine bases and their nucleoside derivatives (Fig. 7).



#### a) Pyrimidine Nucleotide Biosynthesis de novo

The pathway of pyrimidine nucleotide biosynthesis de novo is summarized below (Fig. 8). The enzymes involved are listed in Table 4.



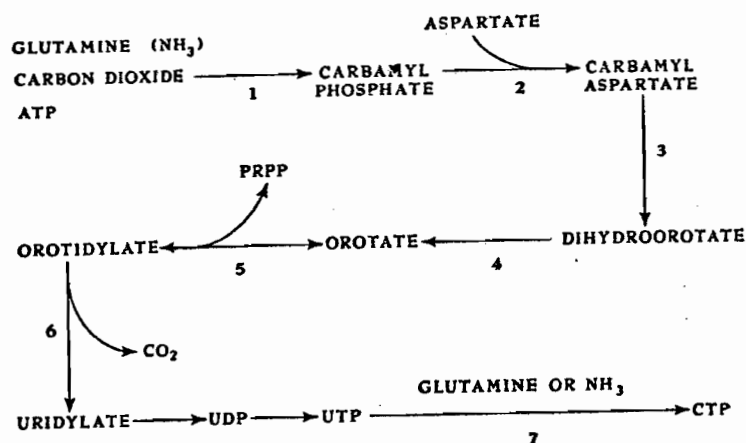


Fig. 8. Pyrimidine nucleotide biosynthesis de novo.  
(from Henderson and Patterson, 1973).

TABLE 4

ENZYMES OF PYRIMIDINE RIBONUCLEOTIDE BIOSYNTHESIS *de Novo*<sup>a</sup>

Enzyme Commission number	Systematic name	Trivial name
2.7.2.5	ATP: carbamate phospho- transferase (dephosphorylating)	Carbamyl phosphate synthetase I
1	—	Carbamyl phosphate synthetase II
2.7.2.2	ATP: carbamate phosphotransferase	Carbamate kinase
2	Carbamoylphosphate: L-aspartate carbamoyl- transferase	Aspartate carbamyltransferase
3	L-4,5-Dihydro-orotate amidohydrolase	Dihydroorotase
4	L-4,5-Dihydro-orotate: oxygen oxidoreductase	Dihydroorotate dehydrogenase
5	Orotidine-5'-phosphate: pyrophosphate phosphoribosyltransferase	Orotate phosphoribosyltrans- ferase, orotidine-5'-phosphate pyrophosphorylase
6	Orotidine-5'-phosphate carboxy-lyase	Orotidine-5'-phosphate decarboxylase
7	UTP: ammonia ligase (ADP)	CTP synthetase

<sup>a</sup> Numbers in first column indicate reaction in summary diagram (Fig. 8)  
(from Henderson and Patterson, 1973).

## b) Pyrimidine Nucleotide Synthesis from Bases and Nucleosides

The pathways are summarized in Fig. 9. The enzymes are listed in Table 5.

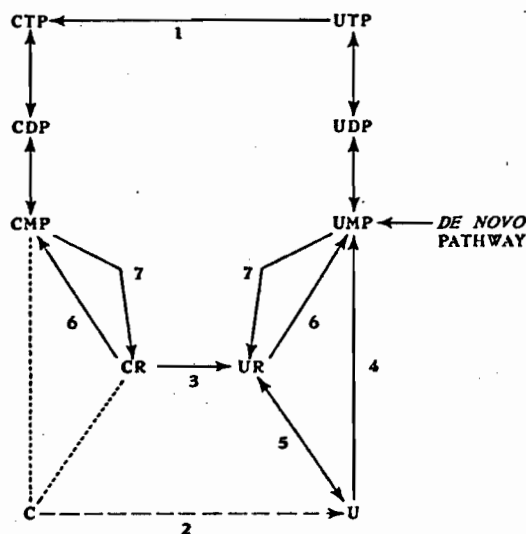


FIG. 9. Metabolism of uracil, cytosine, and their ribonucleosides. Solid lines, enzyme activity in both animal and microbial cells; large-dashed line, enzyme activity in microbial cells only; small-dashed lines, reaction not known.

(from Henderson and Patterson, 1973).

TABLE 5  
ENZYMES OF PYRIMIDINE RIBONUCLEOTIDE SYNTHESIS  
FROM BASES AND RIBONUCLEOSIDES\*

Enzyme Commission number	Systematic name	Trivial name
1. 6.3.4.2	UTP: ammonia ligase (ADP)	CTP synthetase
2. 3.5.4.1	Cytosine aminohydrolase	Cytosine deaminase
3. 3.5.4.5	Cytidine aminohydrolase	Cytidine deaminase
4. —	—	Uracil phosphoribosyltransferase
5. 2.4.2.3	Uridine: orthophosphate ribosyltransferase	Uridine phosphorylase
6. 2.7.1.48	ATP: uridine 5'-phospho- transferase	Uridine-cytidine kinase
7. 3.1.3.5	5'-Ribonucleotide phospho- hydrolase	5'-Nucleotidase
— 2.4.2.1	Purine nucleoside: orthophos- phate ribosyltransferase	Purine nucleoside phosphorylase

\* Numbers in first column refer to reactions in summary diagram (Fig. 9)  
(from Henderson and Patterson, 1973).

### c) Interconversion of Pyrimidine Nucleotides

The pathways by which pyrimidine nucleotides are interconverted are summarized in Fig. 10.

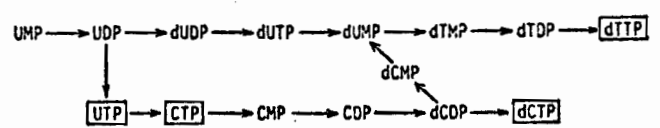


FIG. 10 Normal routes of pyrimidine nucleoside triphosphate formation in animal cells. (from Hauschka, 1973).

It should be noted that deoxythymidylic acid (dTTP) is not synthesized through the de novo biosynthetic pathway but is formed from deoxyuridylic acid (dUMP) by thymidylate synthetase. DNA contains thymine (5-methyluracil) instead of uracil present in RNA. Thymidylate synthetase catalyses the methylation of the uracil moiety of dUMP in a reaction requiring the participation of a folic acid coenzyme,  $N^5, N^{10}$ -methylene tetrahydrofolate, as methyl donor. The enzyme is inhibited by 5-fluoro-deoxyurine (FdUrd) which in its phosphorylated form (FdUMP) acts as a competitive inhibitor of dUMP. In addition to thymidylate synthetase, FdUrd also inhibits thymidine kinase, the enzyme responsible for the formation of dTTP from thymidine (Henderson and Patterson, 1973).

### IV. THE CELL CYCLE

The cell cycle is subdivided on a temporal basis into four main phases:  $G_1$ , S (DNA synthesis period),  $G_2$ , and M (mitosis). The subdivision is based on the observation that cellular DNA is duplicated during a discrete interval in interphase (Howard and Pelc, 1953). Following mitosis there is a gap ( $G_1$ ) in the cycle during which no nuclear DNA synthesis takes place. This phase is followed by a period of DNA synthesis (S)

during which time the DNA content of the interphase nucleus is doubled. The S phase is followed by another gap ( $G_2$ ) in the cycle when again no nuclear DNA synthesis takes place. Finally, the  $G_2$  phase is terminated by mitosis.

The S and M periods are defined by DNA replication and cell division respectively and progress through the cell cycle is usually assessed by observing these two readily identifiable processes. No specific events have been similarly identified that can account for progress of the cell through the  $G_1$  and  $G_2$  periods. The  $G_1$  period is usually assumed to contain a succession of events that leads to the initiation of DNA replication, while the  $G_2$  period is believed to contain a sequence of steps initiated by the completion of DNA synthesis and leading to cell division.

The lengths of S,  $G_2$ , and M are, in general, found to be relatively constant, while the length of  $G_1$  can vary greatly among different cell types (Defendi and Manson, 1963; Petersen, Anderson and Tobey, 1968; Prescott, 1976). The crucial control events for the regulation of genome replication and cell proliferation seem to reside in  $G_1$ . This is supported by the observation that the complete cessation of cell reproduction in tissues is achieved predominantly by the prolonged arrest of cells in the  $G_1$  period. Thus, cells that reproduce extremely slowly (in kidney, liver, pancreas etc.) or the cells in nonrenewing tissues (neurons, skeletal muscle cells) all contain the  $G_1$  amount of DNA. This has been demonstrated with the small lymphocytes of peripheral blood, which ordinarily do not reproduce in vivo, but can be stimulated to reproduce in culture. Every lymphocyte stimulated to divide goes through an S and a  $G_2$  period prior to mitosis (Prescott and Bender, 1962). The discovery that regulation

of the cell cycle occurs primarily by  $G_1$  arrest has led to the introduction of the " $G_0$ " concept to describe the state of the cell in the arrested condition (Lajtha, 1963). The  $G_0$  state is part of  $G_1$ , since it has been localized to that portion of the cell cycle in which the cell contains an unreplicated genome (Baserga, 1968; Baserga, 1969; Padilla et al., 1974). In the  $G_0$  state the cell may be considered to have withdrawn from the cell cycle. This withdrawal is, for most cell types, reversible, and the cell may reenter the  $G_1$  period of the cycle and resume proliferation in the presence of an appropriate stimulus. Such cells exhibit a characteristic delay (a lag period) before entering the S phase (Gelfant, 1977).

Environmental conditions influence the decision of cells whether to initiate DNA synthesis and undergo division or cease to proliferate (Pardee, 1974). Suboptimal growth conditions shift normal cells into quiescence. Such conditions include high cell density (Robinson and Smith, 1976; Skehan, 1976; Temin, 1971) serum limitations (Martin and Stein, 1976; Rubin and Steiner, 1975), limitation of some amino acids (Martin and Stein, 1976; Holley and Kiernan, 1974; Paul and Walter, 1975) or of some other nutrients, such as phosphate, glucose (Holley and Kiernan, 1974; Kamely and Rudland, 1976), or lipids and biotin (Hatten et al., 1977) and the presence of certain drugs (Pardee et al., 1978). The mechanism of density-dependent inhibition of growth is not yet well understood. Factors that are thought to be involved include medium depletion (Dulbecco and Elkington, 1973; Thrash and Cunningham, 1975; Fodge and Rubin, 1975; Hassel and Engelhardt, 1977), limitation of available growth surface (Dulbecco and Elkington, 1973), direct contact interactions (Skehan, 1976; Skehan and Friedman, 1976), a diffusible inhibitory factor released by the cell (Canagaratna and Riley, 1975), and the amount of cell surface area exposed to the growth medium (Robinson and Smith, 1976). Limitation of all the amino acids

simultaneously (Holley and Kiernan, 1974), or of isoleucine (Tobey, 1973), histidine plus glutamine (Kamely and Rudland, 1976) or arginine (Popescu et al., 1975) has been reported to arrest cells in  $G_0$ , although arginine deprivation of CHO cells resulted in an S phase arrest (Weissfeld and Rouse, 1977).

Evidence has accumulated in favor of a restriction or commitment point between  $G_0$  and S. Presumably, once the biochemical event associated with this point has occurred, a cell is irreversibly committed to initiate DNA synthesis and undergo cell division. A growth stimulus, such as serum, can be added to quiescent cells and then removed at various times. After the restriction point, cells proceed to initiate DNA synthesis in the absence of the growth stimulus (Rubin and Steiner, 1975; Brooks, 1976; Bolen and Smith, 1977). Drugs that preferentially inhibit the commitment event can be added at various times after growth stimulation. The restriction point is defined as the point at which the drug is no longer effective in preventing cells from entering S. Thus, based upon drug studies with caffeine, 5-fluorouracil and puromycin, the location of the restriction point was estimated to be midway through the transition between  $G_0$  and S in BHK cells (Pardee and James, 1975), and midway between mitosis and S in Chinese hamster cells (Epifanova et al., 1975). Although a lot of evidence has been accumulated indicating the significance of the  $G_1$  period in the regulation of the cell cycle,  $G_1$ -less cycles have been observed in unicellular organisms, in certain cells within multicellular organisms (both normal and tumorous), and in mammalian cells in culture. Thus, no  $G_1$  period is detectable in the slime mold, *Physarum* (Nygaard et al., 1960), in *Amoeba proteus* (Ord, 1968; Ron and Prescott, 1969) or in the micronucleus of two ciliated Protozoa, *Tetrahymena* (McDonald, 1962) and *Euplotes* (Kimball and Prescott, 1962).

A  $G_1$  period is also absent in very rapidly proliferating cells within multicellular organisms. Thus, the cleavage stages of sea urchin embryos (Fansler and Loeb, 1969; Hinegardner et al., 1964), *Xenopus* embryos (Graham and Morgan, 1966), snail embryos (Van den Biggelaar, 1971), and mouse embryos (Gamow and Prescott, 1970) lack a  $G_1$  period. Neoplastic cells, in general, have cycles with  $G_1$  periods that are shorter than their normal counterparts. Under certain conditions, some lines of Ehrlich Ascites tumor cells proliferate in the peritoneal cavity of the mouse without a detectable  $G_1$  period (Baserga, 1963; Lala and Patt, 1966).

Finally,  $G_1$ -less cycles have been observed in two kinds of mammalian cells in culture. In Syrian hamster fibroblasts, the stimulation of arrested cells with serum subsequently leads to at least one cell cycle that lacks a  $G_1$  period (Bürk, 1970). Robbins and Scharff (1967) have described the cell cycle for a line of Chinese hamster cells (V79) that completely lacks a  $G_1$  period. Apparently, the absence of  $G_1$  in V79 is observed only in cell cultures that are in logarithmic phase of growth. In confluent monolayers a  $G_1$  period appears, probably because of a slowing down in the rate of cell proliferation. This cell line has potential usefulness for answering a number of questions about the significance of the  $G_1$  period and about the control of the initiation of DNA synthesis.

#### The temporal structure of S phase

There appear to be multiple levels for the control of DNA replication once a cell is committed to S phase (Hand, 1978). There is a degree of control at the level of the chromosome in that certain sections are likely to be synthesized at defined times in S phase. Thus, DNA sequences that replicate within the same portion of succeeding S phases (Mueller and Kajiwara, 1965; Brown and Willi, 1969; Plaut et al., 1966; Amaldi et al.,

1973; German, 1964). Also, the average G + C content is higher in DNA synthesized early in S than in DNA synthesized late (Tobia et al., 1970; Bostock and Prescott, 1971), and heterochromatin is always replicated late in S phase (Lima de Faria and Jaworska, 1968). Early-replicating DNA, and possibly some event coupled to its synthesis, is extremely important for passage through the entire S period (Hamlin and Pardee, 1978).

The DNA within a section of a chromosome is divided into multiple replication units, organized into clusters for the purpose of replication. If waves of DNA synthesis during the S period are the result of different numbers of functioning replication units in operation at different times in S, then the implication is that DNA synthesis initiates at certain clusters of replication units at precise times during S.

The initiation of S phase, the rate of DNA synthesis, and the pattern of DNA accumulation through the cell cycle can be measured and compared using total isotope incorporation from pulse labeling and continuous labeling experiments, percent labeled nuclei in autoradiographs of pulse and continuously labeled cells, and DNA fluorescence per cell using the flow microfluorograph (Van Dilla et al., 1969). Close analysis of the pattern of DNA synthesis in S reveals that DNA content increases in a saltatory fashion, and that the early portion of S phase is a period of low net DNA synthesis which may be mistaken for G<sub>1</sub> if methods of measurements other than autoradiography are used (Klevecz, Keniston and Deaven, 1975).



## V. DNA SYNTHESIS

DNA synthesis is a replication process involving the copying of the deoxyribonucleotide sequence of an original template that is not destroyed in the process. The semiconservative mode of DNA replication in viruses and bacteria is supported by a wide variety of experiments, and the direct evidence for semiconservative replication of eukaryote DNA has been summarized by Taylor (1963) and Prescott (1970).

### a) Units of Replication

The study of the synthesis of one DNA molecule by the bacterial cell has provided a model of how a eukaryote may regulate the replication of a large number of DNA molecules. The demonstration by Cairns (1963) that the *E. coli* genome consists of one circular, double-stranded DNA molecule, apparently replicated by DNA synthesis at one site, focused attention on the structural arrangement of DNA in relation to its replication. Jacob et al., (1963) introduced the concept of a basic unit of replication, termed a "replicon", and proposed that the single DNA molecule found in bacteriophage or bacteria behaved as one replicon. The fundamental property of a replicon is that it can itself set up a specific signal system to regulate its own replication.

Evidence that the DNA in a eukaryotic chromosome is divided into a number of replication units came from numerous sources. Autoradiographic studies of metaphase chromosomes from pulse-labeled cells revealed multiple grain clusters in each chromosome suggesting multiple units of DNA replication (Taylor, 1963). By means of DNA fiber autoradiography, Cairns (1996) found such units in mammalian cells to be tandemly arranged on long DNA fibers. Huberman and Riggs, (1968) confirmed this finding of Cairns and showed, in addition, that in most replication units DNA synthesis proceeds

bidirectionally at a rate of DNA replication ranging from 0.5 to 1.2  $\mu\text{m}/\text{min}$ . Thus, the replication unit is a stretch of chromosomal DNA replicated by two adjacent growing points which share a common origin and move in opposite directions from that origin (Huberman and Riggs, 1968). These findings have been confirmed in a number of different eukaryotic species, that is, mammals, avian, amphibian and plants (Lark et al., 1971; Hand and Tamm, 1973; McFarlane and Callan, 1973; Callan, 1974; Van't Hof, 1975).

The size of the replication units has been shown to vary in length between 4  $\mu\text{m}$  (Taylor and Hozier, 1976) and 400  $\mu\text{m}$  (Yurov, 1977) with most values lying between 15 and 100  $\mu\text{m}$  (reviews by Edenberg and Huberman, 1975; Sheinin et al., 1978; Hand, 1978). In general, the size of replication units varies in different cell types with different growth conditions. This was first observed by Callan (1972) and Blumenthal et al., (1974), who found that cells with faster rates of DNA synthesis has smaller replication units.

#### b) Initiation of DNA Replication

Although no simple direct technique exists for measuring the number of origins of DNA replication in mammalian cells they have been estimated to be between 50,000 and 100,000 (Hand and Tamm, 1974; Tamm et al., 1978; Cohen et al., 1978). It appears that not all potential initiation sites are operating during S phase. The evidence comes from numerous studies. The distance between activated initiation sites in DNA of somatic cells of mature *Triturus* are in the range of 100-350  $\mu\text{m}$  or greater (Callan, 1972; 1974), while in the embryos in the neurula stage initiation sites are about 40  $\mu\text{m}$  apart. Similarly, in rapidly replicating cleavage nuclei of *Drosophila melanogaster* initiation sites are closer together than in the slowly replicating nuclei in somatic cell cultures (Blumenthal et al., 1973).

In early embryonic cells the S phase may be very short and the closely situated initiation sites are necessary to accomplish the rapid duplication of the whole chromosome complement. In Chinese hamster cells intervals between initiation sites decrease when cells blocked at the G<sub>1</sub>/S interface by treatment with 5-fluorodeoxyuridine are released into the S phase (Taylor and Hozier, 1976; Taylor, 1977). In these studies, the shortest replication unit length was observed after the longest duration of the block. A similar reduction in the size of active replication units following a reversal of a fluorodeoxyuridine block was also observed by Ockey and Saffhill (1976). In SV40-transformed Chinese hamsters (Martin and Oppenheim, 1977) and BALB/c3T3 (Oppenheim and Martin, 1978) cells initiation intervals are smaller than in untransformed cells. And in polyoma-infected cells there is an increase in the frequency of initiation of cellular DNA synthesis (Cheevers et al., 1972), also consistent with the idea that not all initiation sites for DNA replication are utilized under normal growth conditions in cell cultures.

The physical nature of replication origins in mammalian cell DNA has not been established. Regions of inverted complementary (palindromic) sequences, which are widely distributed in DNA (Davidson et al., 1973; Wilson and Thomas, 1974; Schmidt and Deininger, 1975; Cech and Hearst, 1975), may represent potential initiation sites for DNA replication (Bollum, 1975; Edenberg and Huberman, 1975). Palindromic sequences have been demonstrated near the origin of replication of SV40 DNA (Subramanian et al., 1977; Hsu and Jelinek, 1977) and Adenovirus DNA (Sussenbach and Kuijk, 1978).

### c) Clustering of Replication Units

A common observation in DNA fiber autoradiography experiments has been the coordinated initiation of DNA synthesis on groups of adjacent

replication units - clusters (Huberman and Riggs, 1968; Hori and Lark, 1973; Blumenthal et al., 1973; Hand and Tamm, 1974; Hand, 1975).

Hand (1975a; 1975b; 1977) has shown by means of statistical analysis that clusters of replication units initiate replication in synchrony more frequently than expected on the basis of chance, and they have similar rate of replication for movement. They also tend to be similar in size (McFarlane and Callan, 1973). Clusters of active replication units along DNA molecules have also been observed in numerous other studies using a variety of techniques such as fluorescent dyes (Latt, 1973; Willard and Latt, 1976), X-irradiation (Painter and Young, 1975; 1976), UV-irradiation (Povirk, 1977) and density-labeling (Kowalski and Cheevers, 1976; Planck and Mueller, 1977).

#### d) Discontinuous DNA Synthesis

The DNA polymerases which have been purified from both prokaryotic and eukaryotic cells are capable of elongation DNA in vitro only in the 5' to 3' direction. At each replication fork, one daughter strand must be synthesized with overall polarity 3' to 5'. The discovery by Okazaki (Okazaki et al., 1968) of small primary replication intermediates, (Okazaki pieces) in *E. coli* provided a mechanism by which elongation of both nascent strands could proceed in the 5' to 3' direction in prokaryotes. The existence of these pieces has since been confirmed by others (reviews by Kornberg, 1974; Gefter, 1975) and more recent evidence suggests that a similar process exists in eukaryotes (review by Edenberg and Huberman, 1975; Sheinin et al., 1978; Hand, 1978). In mammalian cells the existence of Okazaki pieces was definitely demonstrated by Huberman and Horwitz (1973) although other investigators before them had reported the presence of such rapidly synthesized, short strands (Schandl and

Taylor, 1969; Painter and Schaefer, 1969; Kidwell and Mueller, 1969; Nuzzo et al., 1970; Sato et al., 1970; Hyodo et al., 1970).

The DNAs of papovaviruses are replicated in the nuclei of their permissive host cells, and with the exception of a viral protein required for the initiation of replication (Tegtmeyer, 1972; Francke and Eckhart, 1973; Chou et al., 1974), all the steps in viral DNA synthesis are carried out by enzymes of the host cell. Papovaviruses, therefore, are a useful probe for studying mammalian DNA replication. The size measurements made on Okazaki pieces indicate that in polyoma, SV40 and mammalian cells, these pieces are approximately 50 - 150 nucleotides long (Fareed and Salzman, 1972; Magnusson et al., 1973; Francke and Hunter, 1974; Hunter and Francke, 1974) sedimenting on alkaline sucrose gradients with a value of about 4S. The corresponding pieces in prokaryotes are approximately 1000 nucleotides long sedimenting with a value of 10S (reviews by Edenberg and Huberman, 1975; Sheinin, 1978; Hand, 1978).

The synthesis of Okazaki pieces is believed to be initiated by the synthesis of a ribonucleotide primer since no known DNA polymerase is able to initiate DNA synthesis directly on a DNA template (Weissbach, 1977). The evidence for such priming comes from experiments involving nearest neighbor frequency analysis which indicate the presence of RNA-DNA junctions in molecules newly synthesized in vitro (Magnusson et al., 1973; Pigiet et al., 1974; Hunter and Francke, 1974; Tseng and Goulian, 1975; Wagar and Huberman, 1975). Other experiments on the molecules synthesized in vitro indicate the presence of 7 - 10 ribonucleotides terminated in a triphosphate at the 5' end of the DNA (Reichard et al., 1974; Kaufman et al., 1977; Tseng and Goulian, 1977). However, there is yet no evidence to indicate the involvement of ribonucleotides in the synthesis of DNA in vivo analogous to that for prokaryotes (Brutlag et al., 1971; Sugino et al., 1972;

Ogawa et al., 1977; Miyamoto and Denhardt, 1977).

There is controversy over whether replication proceeds in a totally discontinuous (i.e., both strands synthesized discontinuously through the formation of Okazaki pieces) or in a semidiscontinuous (i.e., only one strand synthesized discontinuously) fashion. Both prokaryotic and eukaryotic experiments have yielded contradictory results. The evidence for totally discontinuous synthesis of both nascent strands comes from experiments in which more than 50% of the radioactive precursor incorporated into DNA in very short pulses is incorporated into Okazaki pieces (Okazaki et al., 1968; Fareed and Salzman, 1972; Huberman and Horwitz, 1973; Gautschi and Clarkson, 1975; Kurosawa and Okazaki, 1975; Tseng and Goulian, 1975). Additional evidence supporting the discontinuous model of synthesis comes from hybridization experiments in which a significant proportion of the viral Okazaki pieces purified from virus-infected cells is capable of selfannealing (Magnusson, 1973; Fareed et al., 1973; Pigiet et al., 1973). The evidence for the semidiscontinuous model of synthesis, on the other hand, comes from experiments in which the results are contrary to the above. Thus, some investigators have found that a maximum of 50% of pulse-labeled DNA can be detected as Okazaki pieces (Qasba, 1974; Francke and Hunter, 1974; Hershey and Taylor, 1974), while others have found that only a small portion of purified viral Okazaki pieces are capable of selfannealing (Francke and Hunter, 1974; Francke and Vogt, 1975). There are drawbacks to all of these methods of investigation used (Francke and Vogt, 1975; reviews by Edenberg and Huberman, 1975; Hand, 1978) and although the controversy on this point still exists the most recent evidence supports the semidiscontinuous model. Thus, hybridization of Okazaki pieces from replicating papovavirus DNA to restriction enzyme fragments of the viral chromosome from either side of the replication origin shows that approxi-

mately 75% hybridize to restriction fragments of the strand whose overall direction of elongation is 3' to 5' (Perlman and Huberman, 1977; Hunter et al., 1977).

#### e) In vitro DNA Synthesis

The study of DNA synthesis in cell-free systems offers possibilities for certain biochemical and genetic studies which, in intact cells, are made impossible by the inaccessibility of the replication machinery to manipulation. With prokaryotes, one approach to cell-free synthesis has been to permeabilize the cell membrane by treatment with organic solvents or non-ionic detergents, or by plasmolysis. Thus, in vitro replication systems have been successfully used with prokaryotic cells and phages to elucidate specific functions of enzymes and other gene products involved in DNA replication, (reviews by Gefter, 1975; Wickner, 1978). In eukaryotes, various in vitro systems have been described using permeabilized cells (Burgoyne, 1972; Hunter and Francke, 1974; Seki et al., 1975; Berger and Johnson, 1976; Gautschi et al., 1977), isolated nuclei (Lynch et al., 1972; Winnacker et al., 1972; Hershey et al., 1973; Kroken et al., 1975; Tseng and Goulian, 1975), or lysates containing sheared chromatin (Brown and Stubblefield, 1974; Thompson and Mueller, 1975; Brewer, 1975). In most of these, DNA synthesis is semiconservative.

In vitro DNA-synthesizing systems offer the advantage of presenting no significant barrier to exogenous nucleotides or other molecules (such as enzymes and drugs) while retaining varying degrees of the nuclear DNA replication structure. One disadvantage of any in vitro system is that it involves a drastic disruption of cellular structure; therefore, useful information can be obtained from an in vitro system only to the extent that it can be shown to mimic normal in vivo DNA replication. Another

disadvantage of in vitro systems so far developed is that the rate of DNA synthesis is lower than the in vivo rate initially and decreases to zero during incubation over a period of time which varies with the system, (Gautschi et al., 1977; Fraser and Huberman, 1977). Despite these disadvantages, in vitro systems can potentially be used to identify and characterize eukaryotic proteins involved in DNA replication.

#### f) Enzymes involved in DNA Replication

Among all the enzymes known to be involved in DNA replication the best studied are the DNA polymerases. Several different forms of both cytoplasmic and nuclear DNA polymerases have been extracted from higher eukaryotic cells. In mammalian cells three different DNA polymerases can be distinguished, classified as DNA polymerase  $\alpha$ ,  $\beta$  or  $\gamma$  according to their molecular weight, their sensitivity to sulfhydryl inhibitors, and their reactions with DNA template and a variety of primer molecules (Weissbach, 1977). The  $\alpha$  and  $\beta$  polymerase activities are associated with the nucleus (Herrick et al., 1976; Lynch et al., 1975; Foster and Gurney, 1976), while the  $\gamma$  DNA polymerase is closely related to, or identical with the mitochondrial DNA polymerase (Bolden et al., 1977). The  $\alpha$ -polymerase is incapable of using synthetic or natural RNA strands as template (Bollum, 1975; Sedwick et al., 1972), but it can elongate synthetic and natural RNA primers (Spadari and Weissbach, 1975; Keller, 1972; Chang and Bollum, 1972). Unlike the  $\alpha$ -polymerase,  $\beta$ -polymerase is capable of using a synthetic RNA-DNA template-primer combination, but it cannot use natural RNA as a primer although it can, inefficiently, extend a synthetic RNA primer (Bollum, 1975; Spadari and Weissbach, 1975). The  $\gamma$ -polymerase constitutes only about 1% of the total cellular DNA polymerase activity, and like the  $\beta$ -polymerase can use only a synthetic RNA primer (Spadari



and Weissbach, 1975).

Investigations with synchronized tissue culture cells have shown a positive correlation between the rate of DNA synthesis and the activity of the  $\alpha$ -polymerase (Spandari and Weissbach, 1974). The rise in  $\alpha$ -polymerase activity in growing cells or during the S phase of the cell cycle has been generally assumed to indicate that this polymerase is the "replicative" one in DNA synthesis, but this association may have other causes (Weissbach, 1977).

In addition to DNA polymerases, the specific activities of several proteins putatively involved in mammalian DNA replication increase during late G<sub>1</sub>-early S. Thus, it is possible that regulation of DNA replication in mammalian cells might be accomplished, at least in part, by the programmed synthesis of particular replicative proteins. Apart from the DNA polymerases, these proteins are: Thymidine kinase (Brent, Butlér and Crathorn, 1965; Kit et al., 1966); thymidylate kinase and deoxycytidylate kinase (Kit and Jorgensen, 1976); ribonucleotide reductase (Larssen and Reichard, 1967); several DNA binding proteins (Salas and Green, 1971); and unwinding proteins (Champoux and Dulbecco, 1972).

## VI. HISTONE SYNTHESIS AND THE S PHASE

Histone synthesis takes place on small polysomes in the cytoplasm from which completed histone molecules are rapidly transported into the nucleus (Pederson and Robbins, 1970).

The synthesis of histones occurs predominantly during the S period and is apparently tightly coupled to DNA synthesis: in cultures of synchronized cells pulse-labeled histone mRNA appears on polysomes at the beginning of the S phase, increases to a maximum just before the peak of DNA synthesis and then declines (Borun et al., 1967). Also, compounds that inhibit DNA synthesis, such as hydroxyurea and high levels of thymidine, block histone production (Spalding et al., 1966). As a result of hydroxyurea and cytosine arabinoside treatment histone mRNA disappears from the polyribosomes (Gallwitz, 1975; Borun et al., 1975). The loss of histone mRNA during hydroxyurea inhibition of DNA synthesis can be prevented with cycloheximide, implying the synthesis of some kind of regulatory protein induced by the shutdown of DNA synthesis (Gallwitz, 1975).

The absence of detectable mRNA for histones in the cytoplasm of  $G_1$  cells indicates that transcription of histone genes is activated near the  $G_1$ -S border. In HeLa cells blocked by high thymidine, mRNA's for histone synthesis are produced immediately upon release of the block (Breindl and Gallwitz, 1973). Synthesis of histone mRNA continues through S and  $G_2$ . During mitosis or as the cells enter  $G_1$  all histone mRNA is destroyed (Allred et al., 1976).

Although histone synthesis occurs predominantly during DNA synthesis there have been instances in which the two processes have been uncoupled. A low rate of histone synthesis appears to occur in  $G_1$ - or early S-arrested mammalian cells (Appels and Ringertz, 1974; Gurley et al., 1972; Stellwagen and Cole, 1969; Raydt et al., 1977) and in SV40 infected cells in which

host DNA synthesis is inhibited (Liberti et al., 1976).

It has been suggested that histones may be involved in some way with DNA chain elongation in eukaryotic protein (Weintraub, 1972; Woese, 1973). The reduced rate of DNA synthesis would then be a consequence of the availability of only the parental, and no newly synthesized histones. Recent work has shown that histones become associated with nascent DNA within nucleosomal subunits (Elgin and Weintraub, 1975; Kornberg, 1977). Newly replicated DNA, however, is covered with both old and new histones, and newly made histones are distributed at random in the genome (Jackson, Granner and Chalkley, 1975; 1976).

## VII. OVERALL SUMMARY

Eukaryotic DNA replication is an elaborately regulated process involving not only the actual machinery of deoxyribonucleotide synthesis but also the formation of DNA-associated proteins, RNA oligonucleotides and other cellular components. Enzymatic studies have provided evidence for nucleases, polymerases, ligating enzymes, and other proteins which could fulfill these various functions.

The chromosomal DNA in eukaryotes is subdivided for the purpose of replication into multiple discrete units. These linear units are of varying size and contain each a centrally placed initiation point where replication starts and proceeds outward at two fork-like growing points. When forks from adjacent units meet, the newly synthesized DNA chains fuse, producing long strands of chromosomal DNA. The average rate of replication fork movement is  $1 \mu\text{m}/\text{min}$ . Within the replication unit DNA is synthesized discontinuously through the formation of Okazaki pieces on the strand whose overall polarity is 3' to 5'. These short intermediates are synthesized on RNA primers and are subsequently joined to form progeny DNA strands through a series of steps involving the removal of the RNA primer, gap-filling by a DNA polymerase believed to be different from that involved in the synthesis of the short DNA chains and finally ligation.

Chromosomal DNA synthesis is temporally and spatially regulated. There are at least three levels of regulation in eukaryotes: entry of cells into the S phase, initiation of subchromosomal sections and initiation of replication units.

Control of nucleic acid synthesis by precursor nucleotide pool concentration has been frequently proposed in the literature. DNA synthesis, of course, depends on the availability of building blocks, but there is no evidence suggesting that the general supply of deoxynucleotides regulates

DNA synthesis. Studies on synchronized cell populations have revealed increased activity during S phase of numerous enzymes involved in the formation of nucleotide precursors for DNA synthesis. Such studies have also shown that the levels of the dNTP pools are generally very low during  $G_1$  and increase steadily during S, generally reaching a peak after DNA synthesis has peaked. The finding that the dCTP pool most closely follows the course of DNA synthesis has led to the suggestion that this pool might serve a regulatory function, but no specific role has been assigned so far.

Although many advances have been made toward understanding the entire process of DNA synthesis in eukaryotic cells, many steps still need to be elucidated. The biosynthesis of the ribonucleotides and deoxyribonucleotides is a central process for all cells since they are precursors to nucleic acids. An important aspect of the biosynthesis of the nucleotides is the pathway of formation of their bases, the pyrimidines and purines. In the study that follows we present an analysis of the effect of lack of purines on the size of the intracellular dNTP pools, and on DNA synthesis, in an attempt to elucidate the relationship between the purine precursors of the dNTPs and DNA synthesis. Such a relationship has not been studied before at the purine precursor level. Previous studies have analysed the relationship between the dNTP pools and DNA synthesis through interference at the level of the reduction of ribonucleotides to deoxyribonucleotides. Such interference was brought about invariably by the use of drugs that are known to have a multitude of nonspecific effects in addition to the specific biochemical effect attributed to them. Our system has the advantage of dealing with a characterized biochemical defect in the mutant cell line, V79 pur 1, and is thus free of any pharmacologic agents that might cause nonspecific metabolic alterations and

thereby alter the experimental results. The work focuses primarily on the effect of purine deprivation on 1) the size of the intracellular dNTP pools; 2) the ability of the cells to progress through the cell cycle; and 3) on ongoing DNA synthesis.

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CHAPTER 2

EFFECT OF PURINE DEPRIVATION ON DNA SYNTHESIS  
AND DEOXYRIBONUCLEOSIDE TRIPHOSPHATE POOLS OF  
A MAMMALIAN PURINE AUXOTROPHIC MUTANT CELL LINE\*

\*This chapter has been submitted for publication to The Journal of Biological Chemistry (Maria Zannis-Hadjopoulos, Ernst A. Baumann, and Roger Hand).

ABSTRACT

When the V79 pur 1 cell line, a purine auxotrophic mutant of Chinese hamster lung fibroblasts, is maintained in the absence of purines, the cells stop growing but remain viable for up to 48 h. Within the first 6 h after purine deprivation, there is a rapid decrease in the incorporation of  $^3\text{H}$ -thymidine into acid-precipitable material as well as a decrease in the fraction of cells incorporating thymidine. This inhibition is due to arrest of cells in the DNA synthesis phase of the growth cycle, as shown by flow microfluorographic analysis of the cellular DNA content. Analysis of the intracellular deoxyribonucleoside triphosphate (dNTP) content shows an expansion of the pyrimidine dNTP pools and a decrease in size of the purine dNTP pools. The largest pool variations are exhibited by dCTP, which increases to twice the control level, and by dATP which decreases to 34% of the control level within 6 h after purine depletion. Studies of in vitro DNA synthesis show that the purine-depleted cells maintain an intact replication apparatus as evidenced by normal  $^3\text{H}$ -dTTP incorporation in the presence of exogenously supplied triphosphate precursors. Our results indicate that the inhibition of DNA synthesis caused by the block to purine synthesis in this mutant may be bypassed by restoring the nucleotide pools, and suggest that the size of these nucleotide pools may be a critical factor in DNA synthesis.

V79 pur 1 is a purine auxotrophic mutant of the Chinese hamster lung cell line, V79, that has been isolated through treatment with 5-bromo-deoxyuridine and black light (5). Feldman and Taylor (7), in characterizing the defect, found that these cells were deficient in phosphoribosylpyrophosphate amidotransferase (E.C.2.4.2.14), the first enzyme in the de novo purine biosynthetic pathway. They also found that, when grown in the absence of exogenous purines, the cells show a rapid and sharp decline in the incorporation of labeled precursors into nucleic acids that decreases within 6 h to less than 10% of the level of cells grown in the presence of purines. Overall protein synthesis is affected to a lesser extent and the decline is more gradual (31).

The relationship between deoxyribonucleoside triphosphate (dNTP) pool sizes and DNA synthesis has been extensively investigated (3,15,16, 18,30,33,34). Generally, dNTP pools increase in size just before initiation of DNA synthesis and attain maximal levels during the S phase (9,18,28,34). Interference with the dNTP pools through treatment with drugs results in marked changes in the rates of DNA synthesis. Hydroxyurea causes a severe reduction in DNA synthesis through inhibition of ribonucleotide reductase (E.C.1.17.4.1; ribonucleoside diphosphate reductase), the enzyme responsible for converting ribonucleotides to deoxyribonucleotides (36). This inhibition of DNA synthesis is paralleled by a depletion of the intracellular purine dNTP pools, dGTP and dATP (20,27,29), or of dATP only (34), with the exception of mouse L929 cells, where the size of the dNTP pools remains unchanged (1). Treatment of cells with high concentrations of thymidine, on the other hand, causes a specific depletion of the dCTP pool and a parallel inhibition of DNA synthesis which can be reversed upon restoration of normal dCTP pool levels (3). This finding led to the suggestion that dCTP pool may serve a regulatory function in DNA synthesis.

There are drawbacks to all studies in which pharmacologic agents are used to inhibit cellular metabolism, the most obvious of which is that the agents may have effects in addition to the specific biochemical inhibition usually attributed to them (6). Hydroxyurea, for example, causes cytopathic effects in cells that cannot be linked solely to the inhibition of ribonucleotide reductase (19,25,32).

In the present study, we have taken advantage of the knowledge of the precise biochemical defect in the V79 pur 1 cell line to study the effect of purine deprivation on the cellular dNTP levels and DNA synthesis. Our results indicate that it causes a reduction of purine dNTP pools associated with an expansion of pyrimidine dNTP pools and a marked inhibition of ongoing DNA synthesis. Restoration of adequate pool size either in vivo or in vitro in a lysed cell system causes a rapid resumption of precursor incorporation into DNA.

#### EXPERIMENTAL PROCEDURES

Cells - The cell lines V79 pur 1 and V79 were kindly supplied to us by Dr. M.W. Taylor, University of Indiana. The V79 pur 1 cells were maintained in Ham's F10 medium (8) supplemented with 5% fetal calf serum (FCS), 0.25  $\mu\text{g}/\text{ml}$  amphotericin B, 50  $\mu\text{g}/\text{ml}$  gentamicin, and 60  $\mu\text{g}/\text{ml}$  tylocine. The parent line, V79, was maintained in Minimal Essential Medium (MEM) supplemented with 5% FCS and antibiotics. Cells were grown in 75  $\text{cm}^2$  tissue culture flasks (Falcon) at 37° C and transferred 1:4 when confluent. They were checked periodically for mycoplasma contamination by whole cell autoradiography and by electron microscopy, as described by Schneider (24), and found to be negative.

Experimental Conditions - For experiments, unsynchronized cells in logarithmic growth were used. They were transferred on the preceding day to plastic petri dishes at a density of  $2 \times 10^5$  cells/dish and maintained overnight in F10 medium. To deplete the cells of purines and produce restrictive growth conditions, the medium was replaced with MEM supplemented with 5% dialyzed FCS. Control cells received the same medium to which hypoxanthine ( $30 \mu\text{M}$ ) was added. As additional controls in some experiments, cells received F10 supplemented with 5% FCS as the replacement medium. Cell counts were performed on triplicate samples using a hemocytometer. Two independent counts per plate were performed and the variation in counting was less than 10%. At the same time, viability was determined by trypan blue exclusion.

Incorporation of  $^3\text{H}$ -thymidine - Cells were prelabelled overnight with  $^{14}\text{C}$ -thymidine ( $0.005 \mu\text{Ci/ml}$ ;  $40\text{-}60 \text{ mCi/mmol}$ ). On the day of the experiment, the radioactive medium was removed, the cells were washed with prewarmed phosphate buffered saline (PBS) and were subsequently incubated in the appropriate medium. At the desired time points, the cells were pulse-labeled with  $^3\text{H}$ -thymidine ( $10^{-6} \text{ M}$ ;  $40\text{-}60 \text{ Ci/mmol}$ ) for 15 min at  $37^\circ \text{ C}$ . The cells were then washed twice with ice-cold PBS and lysed with  $0.4\text{N}$  NaOH. To the lysates was added an equal volume of  $1.5\text{N}$  HCl containing 6% sodium pyrophosphate and they were kept on ice for 10-15 minutes. Acid precipitable material was collected on glass fiber filters (Whatman GF/A), washed with cold  $0.1 \text{ N}$  HCl containing 0.5% sodium pyrophosphate, dried by washing with ethanol and acetone and counted in a Beckman LS-8000 scintillation counter. Incorporation of  $^3\text{H}$ -thymidine is expressed either as  $^3\text{H}:^{14}\text{C}$  ratio or as percent incorporation of the control cells.

Whole Cell Autoradiography - Cells grown on sterile 22 mm coverslips were pulse-labeled for 15 min with  $^3\text{H}$ -thymidine at the desired time points, as described above. Incorporation was stopped by washing with ice-cold PBS. The cells were subsequently fixed in ethanol: acetic acid (3:1) and dehydrated by successive washes in 70% and 95% ethanol. The coverslips were then air-dried, mounted on glass slides and coated with NTB-2 emulsion (Eastman Kodak Co.). After exposure for 10 days to 1 month, they were developed, stained with Giemsa, and examined in a light microscope for determination of the labeling index.

Cytofluorographic Analysis of DNA Content - The distribution of cellular DNA content was analysed by flow microfluorography (FMF) as described by Krishan (11). Cells were stained with cold propidium iodide solution (0.05 mg/ml in 0.1% sodium citrate) for 10 min in the dark. Cell fluorescence was measured within 48 h as described by Nishimoto et al (17).

The distribution of cells in the various phases of the cell cycle was obtained from the fluorescence profiles (2). Assuming a symmetrical distribution of cells about the  $G_1$  (channel 25) and  $G_2 + M$  (channel 50) peaks, the fraction of cells in  $G_1$  and  $G_2 + M$  was estimated for each time point by measuring the areas lying to the left of the  $G_1$  peak and right of the  $G_2 + M$  peaks and multiplying by 2. The fraction of cells in S was then determined by subtracting the sum of the above two from the total area. All areas were measured with a microprocessor based planimeter, which is a magnetostrictive tablet with a resolution of 0.1 mm absolute, and the algorithm was measured using integer arithmetics and trapezoidal integrations.



Analysis of Deoxyribonucleotide Pools - Cellular deoxyribonucleoside triphosphate pools were extracted using ice-cold 60% methanol according to Skoog (26). Enzymatic analysis of the pools was performed using *E. coli* DNA polymerase 1 and the synthetic templates poly [d(I-C)] and poly [d(A-T)], as described previously (12,26). The results are expressed as picomoles per  $10^6$  cells.

In Vitro DNA Synthesis - The lysed cell system of Reinhard et al. (23) was used. Cells were partially lysed with 0.01% Brij-58 and subsequently incubated at 37° C in a reaction mixture containing  $^3\text{H}$ -dTTP, an ATP generating system, and an adequate supply of all four ribo- and deoxyribonucleoside triphosphates. The samples were analyzed either for  $^3\text{H}$ -dTTP incorporation into acid precipitable material or for labeling index of the nuclei using the procedures described above.

## RESULTS

Cell Growth - The effect of purine deprivation on growth of the V79 Pur 1 cell line is shown in Fig. 1. During a period of 48 h, the cells in the purine-deprived medium fail to double, although some cell division does occur, conceivably of the cells that had completed S at the time of the shift to depleted medium. In the same time interval, the cells growing in the medium supplemented with hypoxanthine divide with a doubling time of 30 h, while those maintained in F10 medium divide with a doubling time of 24 h. During this period of time, 96-99% of the control and depleted cells remain viable, as determined by trypan blue exclusion. Wild type V79 cells have a doubling time of 22 h in MEM with or without a purine supplement.

DNA Synthesis - We next examined the effect of purine deprivation in the V79 pur 1 cell line on  $^3\text{H}$ -thymidine incorporation into acid insoluble material. The results are shown in Fig. 2a. After 4 h in purine-deprived medium, incorporation drops to 4% of the value in control cells with access to purines, and remains at that level for at least 7 h. Addition of hypoxanthine to cultures that have been deprived of purines for 4 h, when incorporation is at its lowest point, results in an increase in incorporation which reaches 80% of the control level within 1 h. A similar rapid restoration of DNA synthesis is obtained upon addition of hypoxanthine to cells deprived of purines for 5, 6 or 7 h (Table 1, Appendix). Prolongation of purine deprivation up to 23 h results in an increase in incorporation which reaches about 24% of the control rate. Further incubation in the absence of purines past 23 h results in a new decline in incorporation (6% of the control), which stays at that level until cells die after 48 h (Table 2, Appendix). The increase in incorporation of thymidine at 23 h has also been reported by Sriram and Taylor (31), who showed that it is a result of repletion of the intracellular purine pools from breakdown of ribosomal RNA.

Exposure of the cells to FdUrd 30 min before the pulse results in more  $^3\text{H}$  incorporation than in its absence. This difference can probably be attributed to a dilution of the specific radioactivity in depleted cells not exposed to FdUrd, since in these cells, an expansion of the dTTP pool is observed (150% of the control level; see dTTP pool results). Addition of FdUrd, which inhibits the enzyme thymidylate synthetase, counteracts this effect by depleting the endogenous dTTP pool. Incorporation studies using the wild type cell line, V79, revealed a pattern similar to that of the mutant grown in MEM supplemented with hypoxanthine (Table 3, Appendix).

The fraction of cells engaged in DNA synthesis, as determined by whole cell autoradiography (Fig. 2b), shows a very similar time course of decline as thymidine incorporation into acid-precipitable material. Relative values for the fraction of labeled cells are higher than those for thymidine incorporation, and addition of hypoxanthine after 4 h of purine-deprivation results in a return to control levels within 1 h. Again, higher values are obtained in the presence of FdUrd. These findings show that DNA synthesis is rapidly inhibited and restored upon removal and addition of purines, and suggest that purine deprivation induces a block to S phase, although a G<sub>1</sub> block cannot be completely excluded.

Cell Cycle Distribution - To differentiate more reliably between G<sub>1</sub> and S phase block, flow microfluorography was used. Purine-depleted and control cells were treated with propidium iodide and the DNA content of individual cells measured by fluorescence. The results are shown in Fig. 3, in which cells with a G<sub>1</sub>-DNA content are distributed in a peak about channel 25, and those with a G<sub>2</sub> content about channel 50.

In the sample taken at time zero (Fig. 3a), when the cells were in the rich F10 medium, the majority of cells are distributed in S phase. It has previously been reported that several Chinese hamster cells lines, including two sublines of the V79 line, lack a measurable G<sub>1</sub> or G<sub>2</sub> phase or both (14,21). Under optimum growth conditions, such as in F10 medium, V79 pur 1 cells appear to have short G<sub>1</sub> and G<sub>2</sub> phases.

There is an increase in the proportion of cells with a  $G_1$  DNA content when the cultures are switched from the rich F10 medium to MEM containing 5% dialysed FCS (Fig. 3b-k). The redistribution is similar in cells maintained in the presence and absence of supplemental hypoxanthine. This effect therefore, can be attributed to the difference in the medium and the adjustment of cells to less than optimal growth conditions in MEM.

Thus, during the first 6 h of the experiment, the distribution profiles of the cellular DNA content are essentially the same in the purine-deprived and in the control cells (Fig. 3b-3g), although during the same period of time DNA synthesis has been markedly inhibited in the purine-depleted cells. At 20 h, however, a difference can be seen. In the deprived cells (Fig. 3i) the majority of the cells are distributed between channels 20 and 35, that is between  $G_1$  and S, mainly the early part of S, as suggested by the skewness of the distribution. The control cells (Fig. 3h) are distributed more broadly between channels 20 and 60 with the majority of cells in S. Addition of hypoxanthine to cells deprived of purines for 4 h does not significantly alter the DNA distribution profiles (Fig. 3j and 3k) although it rapidly restores DNA synthesis (see previous section).

In Table I, a comparison is made between the percentage of cells in S, as determined by autoradiography (Figure 2) and by microfluorography (Figure 3). The estimate of cells in S based on the FMF data is a minimum one since it does not include those cells in early and late S whose DNA content is almost identical to that of cells in  $G_1$  and  $G_2$  (10,11). The percentage of radioactively labeled cells decreases steadily in the purine deprived cultures over a period of 6 h, while the percentage of

cells with a DNA content characteristic of the S phase does not show a significant change. A decrease in S phase cells, as measured by FMF, occurs after 20 h in the deprived medium.

The lack of alteration in the cell cycle distribution in the face of inhibition of DNA synthesis in the first 6 h of purine deprivation indicates that the deprived cells are arrested in S phase. By 20 h, an enrichment of the G<sub>1</sub> population is observed, apparently caused by cells that had already completed S at the time of purine withdrawal from the medium and proceeded normally through G<sub>2</sub> and M. This is also evident from the growth curve of the mutant in the absence of purines (see Fig. 1) where a slight increase in the number of cells is found at 20 h.

dNTP Pools - Since the mutation in this cell line stops the de novo synthesis of purines, one of the primary effects of exogenous purine deprivation should be a change in the intracellular dNTP pools following removal of purines from the growth medium. We therefore measured the size of the dNTP pools using the E. coli DNA polymerase assay (12,26). The results are shown in Fig. 4.

Both the dGTP and dATP pools decrease in size, reaching 60% and 34% of the control levels, respectively, following 6 h of purine deprivation. In contrast, the pyrimidine dNTP pools expand during the same period in the deprived medium, with the dCTP pool increasing to twice the control level within 2 h of purine starvation and then decreasing slightly by 6 h, although still remaining above normal levels. The dTTP pool increase is not as marked and its size fluctuates less throughout the course of the experiment, but it also remains above normal levels in the purine-deprived medium. Prolongation of purine deprivation up to 20 h causes a

reversal of these trends and brings all the values closer to control levels probably as a result of breakdown in ribosomal RNA (31). Addition of 30  $\mu$ M hypoxanthine to the depleted medium after 4 h also restores pool sizes toward the normal.

The expansion of the pyrimidine dNTP pools observed above is similar to that seen when the purine dNTP pools are depleted through treatment with hydroxyurea (27,29) and can probably be attributed to altered allosteric regulation of the ribonucleotide reductase activity caused by a relatively low concentration of dATP, which acts as a feedback inhibitor on the enzyme, according to the model described by Reichard (22).

It should be noted that under all experimental conditions (depleted and control), the dCTP pool is the largest and dGTP the smallest, with the dGTP pool measuring less than 5% of the dCTP pool, while the dATP and dTTP levels are intermediate. This finding is consistent with previously reported results with a variety of cell lines (4,9,15,18,28-30,34).

Analyses of dNTP pools were also done on the wild-type cell line, V79, and the values obtained are similar to those of the mutant grown in the purine supplemented medium (Table 4, Appendix).

In Vitro DNA Synthesis - In order to determine whether the inhibition of DNA synthesis observed in vivo under the purine-deprived conditions is a direct result of the decrease in the purine pools, a subcellular DNA synthesizing system was used in which all four deoxyribonucleoside triphosphates were supplied. The cells were deprived of purines in vivo for 6 h and were then partially lysed with Brij-58 and incubated in the reaction mixture containing all four dNTPs. The rate of  $^3\text{H}$ -dTTP incorporation into acid insoluble material is shown in Fig. 5. Within 5

minutes of incubation in vitro, the cells previously deprived of purines incorporate  $^3\text{H}$ -dTTP at a rate of about 70% of the control, which by 60 minutes increases to about 80% of the control. The corresponding in vivo value is 5-10% as measured by  $^3\text{H}$ -thymidine incorporation into acid-insoluble material.

The slight inhibition in  $^3\text{H}$ -dTTP incorporation seen in the cells that were deprived of purines before lysis for in vitro synthesis is not due to fewer cells capable of DNA synthesis. Table II indicates that almost 70% of the cells that had been deprived of purines in vivo synthesize DNA in vitro, whether incubation with radioactive label is in the first or the second half of the one hour reaction. The corresponding control figure is 58%. The slightly higher fraction of labeled nuclei in cells that had been deprived of purines is consistent with the interpretation that these cells are arrested in the S phase. The percentage of cells synthesizing DNA in vivo following 6 h of purine deprivation is 33%. We detect no abnormality in depleted cells when the pools are supplied exogenously to an in vitro system. Therefore, purine depletion in the mutant has little or no effect on the DNA replication enzymes.

#### DISCUSSION

Our results show that purine deprivation of the purine auxotrophic mutant cells, V79 pur 1, causes an inhibition of ongoing DNA synthesis and disrupts traverse of the cell cycle by preventing normal completion of the S phase. Purine deprivation also causes an upset in the balance of the deoxyribonucleoside triphosphate pools, with a decrease of the purine dNTP pools and an expansion of the pyrimidine dNTP pools. Supply of all four dNTPs in equal amounts in vitro leads to resumption of normal precursor incorporation into DNA.

Arrest of cells in the S phase during the first 6 h of purine deprivation is evident by autoradiography, which shows a steady decrease in the number of cells actively engaged in DNA synthesis, while at the same time flow microfluorographic analysis reveals that during this period the proportion of cells with a DNA content characteristic of S remains constant. An alternate explanation might be that the increase in the endogenous dTTP pool under purine-deprived conditions may inhibit phosphorylation of  $^3\text{H}$ -thymidine through feedback inhibition of thymidine kinase. This explanation is less likely since FdUrd treatment - which should block any increase in the dTTP pool - does not prevent the decrease in labeled nuclei in the purine-deprived cells. Additional evidence against an inhibition of thymidine kinase as the sole explanation for the decrease in  $^3\text{H}$ -thymidine incorporation in purine-deprived cells comes from DNA fiber autoradiography experiments, in which normal labeling of DNA fibers is observed and from density labeling experiments which show that residual DNA synthesis is semiconservative<sup>1</sup>. Also, the accumulation of cells in  $G_1$  after 20 h of purine depletion suggests that cells in  $G_2$  were able to continue cycling up to the  $G_1/S$  interface, but were unable to synthesize DNA.

Evidence in support of an S phase block also comes from the observation that when the inhibited cells are provided with hypoxanthine, DNA synthesis resumes at a normal rate within one hour, and the results of in vitro experiments in which 70% of the nuclei from depleted cells become labeled within 30 minutes of incubation in the presence of all four deoxyribonucleoside triphosphates. Finally the observed sizes of all four dNTP pools also argue in favor of an S phase arrest, since they are well above those reported for  $G_1$  populations from a variety of mammalian cell lines, such as human lymphocytes (16,33), secondary mouse embryo cells (18) and Chinese hamster cells (34).



The alterations in the size of the deoxyribonucleoside triphosphate pools in the purine-deprived V79 pur 1 cells are consistent with the genetic defect of these cells. However, the purine dNTP pools, though appreciably decreased over the 6 h period of the experiment, remain always at a measurable size. The intracellular purine ribonucleoside triphosphate (rNTP) pools also remain at a measurable level and show a 5-fold decrease after 12 h of purine deprivation (31). Considering that the shutdown of the de novo purine biosynthetic pathway is complete by 2 h in the absence of exogenous purines (6), we conclude that the purines giving rise to the purine nucleotide pools measured after longer periods of depletion come from the salvage pathways operating in the cells, which utilize preformed purine bases. Salvage synthesis, in addition to de novo synthesis, plays an important role in mammalian cells for maintenance of purine nucleotide levels (13,35), and is catalysed mainly by hypoxanthine-guanine phosphoribosyl transferase (HGPRT), which functions normally in the V79 pur 1 cells.

Although the absolute values of the four dNTP pools in V79 pur 1 cells are altered by purine deprivation, the relationship between them remains unchanged, with the dGTP pool always less than five percent of the dCTP pool, and the dTTP and dATP pools being always of intermediate size. Previous work with hydroxyurea-treated mammalian cells has shown a correlation between the decrease in size of the dGTP pool and inhibition of DNA synthesis (29). In our study, although a reduction in the size of the dGTP pool is observed, it is not as marked as that exhibited by the dATP pool, which we have found to correlate best with the inhibition of DNA synthesis in our cells. Maximum inhibition of DNA synthesis is observed at 6 h post-purine depletion, when the size of the dATP pool is

at a minimum, both values amounting to approximately 30% of the control, while the dGTP pool size fluctuates less and decreases only to 60% of the control. These results are in accordance with those reported for hydroxyurea-treated Chinese hamster cells (34). Although these findings point to a possible positive correlation between the size of the dATP pool and DNA synthesis, the evidence is not conclusive since variation is observed to a greater or lesser extent in all four dNTP pools under conditions of purine starvation. Our in vitro studies suggest that the size of dNTP pools is critical for DNA synthesis of V79 pur 1 cells, since preincubation of the cells in vivo in purine-deprived medium does not reduce  $^3\text{H}$ -TTP incorporation into DNA in a system, in which all four dNTPs are present. Thus it is possible that a normal rate of DNA synthesis depends on the presence of all four dNTPs in balanced amounts.

Finally, although no major discrepancies were noted between our results and those obtained in systems using hydroxyurea, our system has the definite advantage of dealing with a defined biochemical alteration in a mutant cell line and is, therefore, free of any unknown actions of a pharmacologic agent that might also interfere with and alter the experimental results.

Acknowledgements - We thank Dr. Mark Meuth for his advice on experiments and for his critical appraisal of the manuscript. We also thank Dr. Eric Eilen for his assistance with the flow microfluorography, and Dr. Claudio Basilico for allowing us to use the cytofluorograph facilities in his laboratory. This work was supported by grants from the Medical Research Council of Canada and The National Cancer Institute of Canada and has been submitted in partial fulfilment of the requirements for the Ph.D. degree from McGill University for M.Z.-H.

Footnotes

<sup>1</sup> page 7:5; see data presented in Chapter 3.

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TABLE I

S phase cells in V79 pur 1 following purine depletion

Time h	S phase cells			
	Autoradiography <sup>a</sup>		Flow microfluorography	
	Control	Depleted	Control	Depleted
0	71	-	55	-
2	57	48	53	58
4	54	25	48	50
4, repleted 1 h <sup>c</sup>	58	57	NA <sup>b</sup>	44
4, repleted 2 h <sup>c</sup>	63	57	50	52
6	63	33	50	45
20	74	72	55	36

<sup>a</sup> Cells were treated with FdUrd ( $10^{-6}$ M) for 30 min before the radioactive pulse in order to deplete the endogenous dTTP pool and thereby prevent a dilution of the radioactivity.

<sup>b</sup> Not available.

<sup>c</sup> Cells depleted of purines for 4 h and then supplemented with hypoxanthine (repleted).



TABLE II

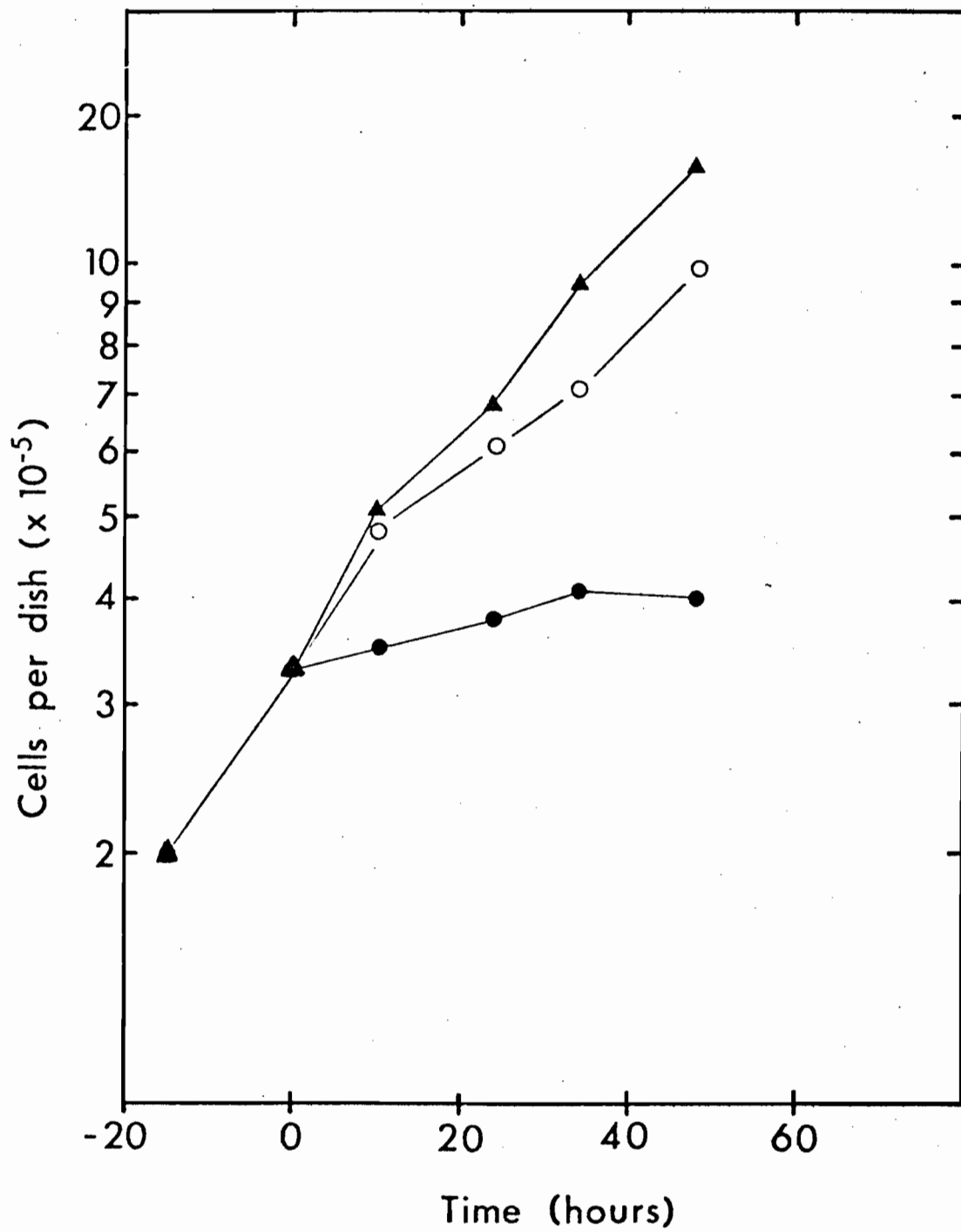
Effect of purine deprivation on the fraction of  
V79 pur 1 cells synthesizing DNA in vitro

Cells deprived of purines for 6 h in vivo were partially lysed with Brij-58 and pulse-labeled with  $^3\text{H}$ -dTTP ( $10^{-5}$  M; 20  $\mu\text{Ci/ml}$ ) at 37° C for various time intervals. The labeling index was determined after the nuclei were spread on glass slides and subjected to autoradiography.

Labeling time in vitro	Labeled nuclei	
	Control	Depleted
min		%
0 - 30	58	69
30 - 60 <sup>a</sup>	58	69

<sup>a</sup>From 0 - 30 min the samples were incubated in the presence of unlabeled dTTP ( $10^{-5}$  M).

FIG. 1. Effect of different culture media on the growth of V79 pur 1 cells. After growth overnight in F10 + 5% FCS, the cells were divided into 3 replicate sets which received F10 + 5% FCS (▲), MEM + 5% dialysed FCS + 30  $\mu$ M hypoxanthine (○), or MEM 5% dialysed FCS (●). At the time points indicated, cells were counted and tested for viability by trypan blue exclusion.



5

FIG. 2. Effect of purine deprivation on  $^3\text{H}$ -thymidine incorporation into acid insoluble material (a), and on the fraction of cells synthesizing DNA (b). Cells were seeded into petri dishes in F10 + 5% FCS. After growth overnight, half the dishes received medium without purines and the other half received medium supplemented with  $30\ \mu\text{M}$  hypoxanthine (control). At the time points indicated, samples were pulsed with  $^3\text{H}$ -thymidine ( $1\ \mu\text{Ci ml}$ ) for 15 min. FdUrd ( $10^{-6}\ \text{M}$ ) was added to an additional set of dishes 30 min before the radioactive pulse. The data are expressed as percent incorporation of the corresponding control cells maintained in hypoxanthine-supplemented medium. (•), V79 pur 1 in purine-depleted MEM with FdUrd; (◦), V79 pur 1 in purine-depleted MEM without FdUrd; (▲), V79 pur 1 deprived of purines for 4 h and then supplemented with  $30\ \mu\text{M}$  hypoxanthine (arrow). CPM incorporated into acid-precipitable material were normalized to  $\text{CPM}/10^6$  cells.

6

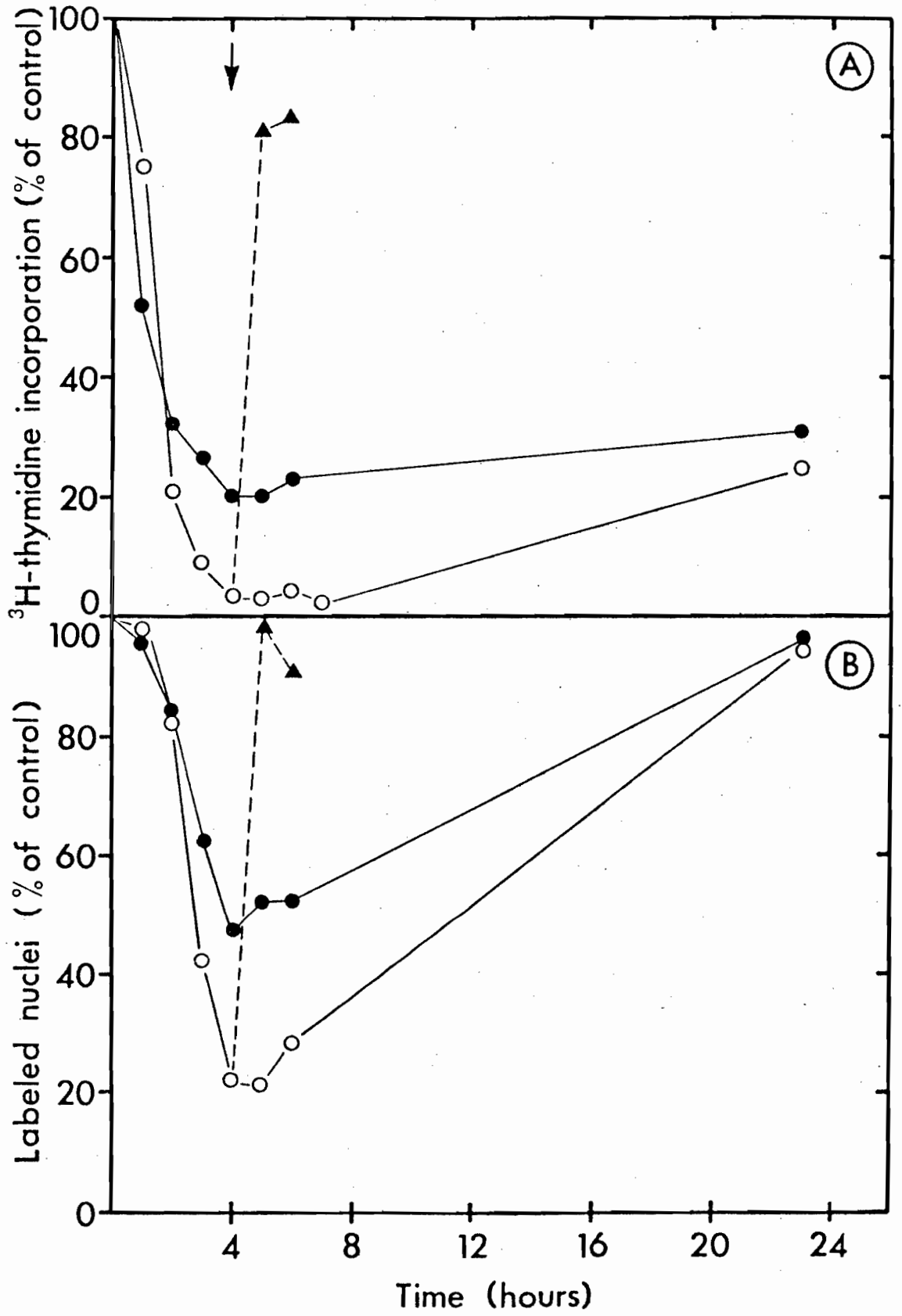


FIG. 3. Effect of purine deprivation on cell cycle distribution of V79 pur 1 cells. After growth overnight in F10 + 5% FCS, half the dishes received purine-deprived medium and the other half received medium supplemented with 30  $\mu$ M hypoxanthine. At the time points indicated the cells were stained with propidium iodide and prepared for FIF analysis. Channel 25 corresponds to a DNA content characteristic of G<sub>1</sub>. C indicates purine supplemented cells (control), D indicates purine deprived cells and re indicates purine repleted cells.

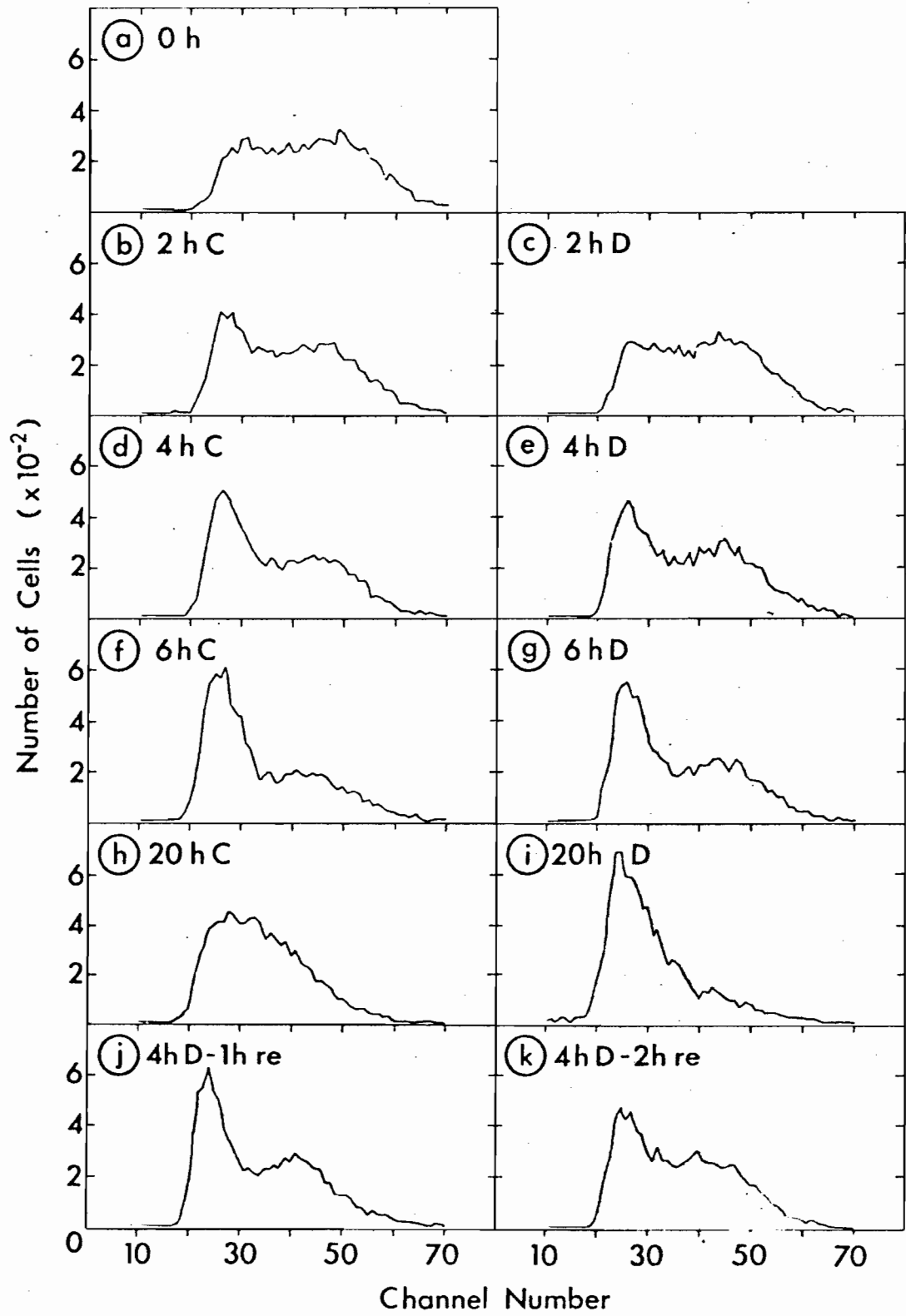


FIG. 4. Effect of purine deprivation on deoxyribonucleoside triphosphate (dNTP) pools in V79 pur 1 cells. Following overnight growth in complete (F10) medium the cultures were shifted to purine-deprived or purine-supplemented medium and the dGTP (a), dATP (b), dTTP (c), and dCTP (d) pools were determined at time points between 0 and 20 h. Please note differences in scales. ( • ), purine-deprived cells; ( o ), cells supplemented with 30  $\mu$ M hypoxanthine (controls); (  $\blacktriangle$  ), cells deprived of purines for 4 h and then supplemented with hypoxanthine (repleted). The arrow indicates time of hypoxanthine addition.



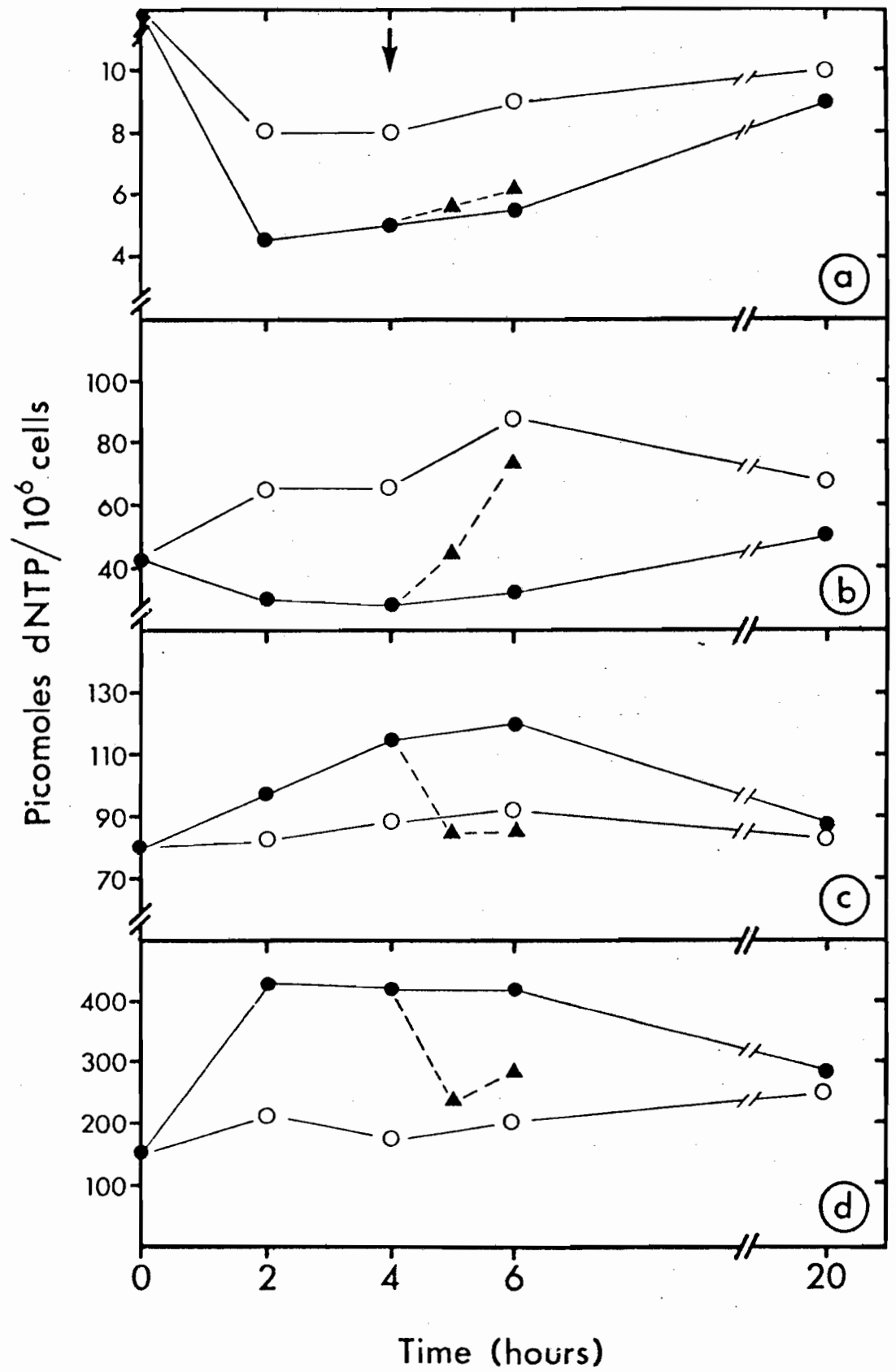
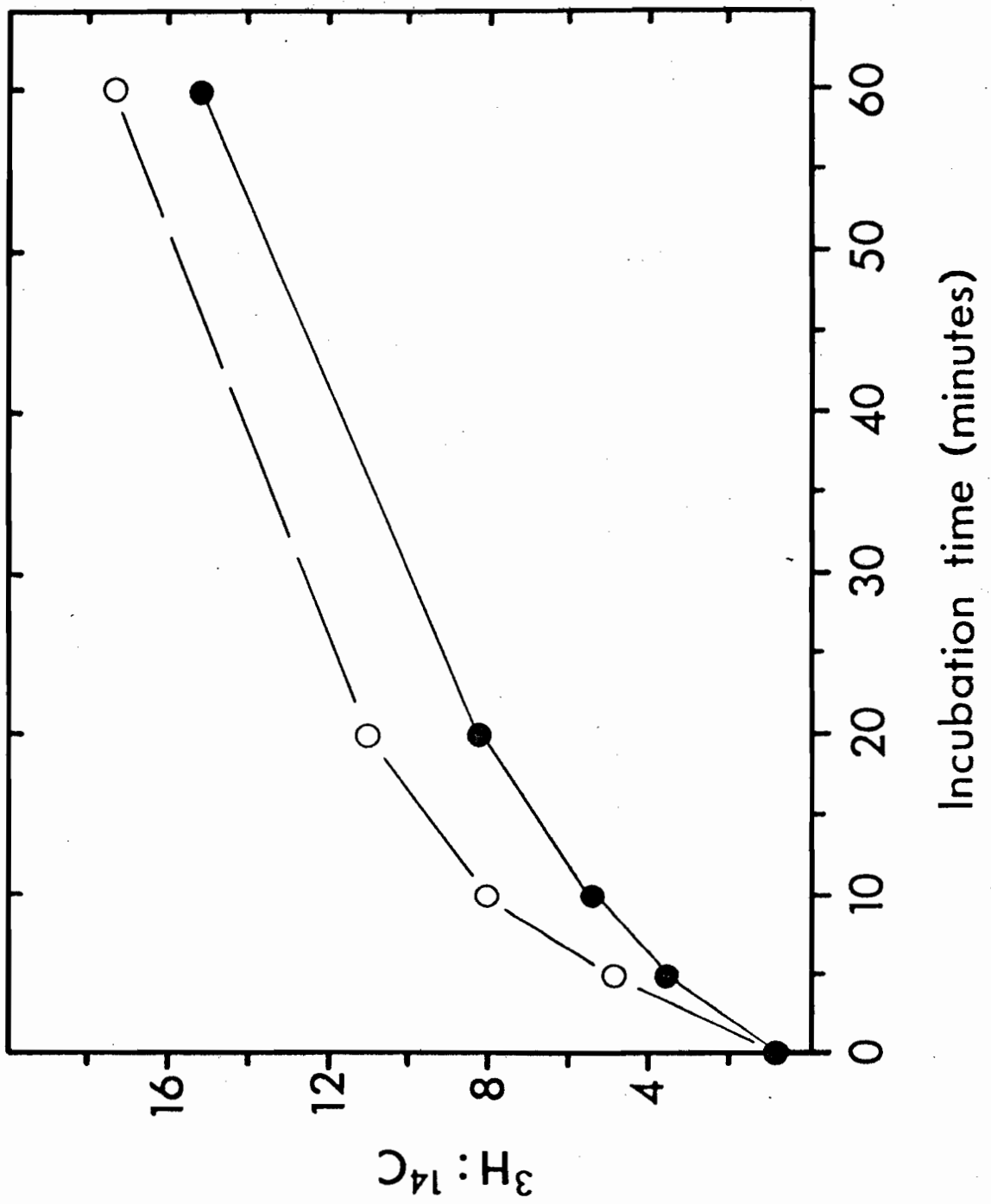


FIG. 5. In vitro DNA synthesis in purine-deprived V79 pur 1 cells.  $^{14}\text{C}$ -prelabeled cells were deprived of purines in vivo for 6 h and subsequently lysed with Brij-58 and incubated in a reaction mixture for in vitro DNA synthesis containing  $^3\text{H}$ -dTTP ( $10^{-5}$  M; 20  $\mu\text{Ci/ml}$ ). At the time points indicated, duplicate samples were removed and the amount of  $^3\text{H}$ -dTTP incorporated into acid-insoluble material was determined. The results are expressed as  $^3\text{H}: ^{14}\text{C}$  ratios. (•), purine-deprived cultures; (◦), cultures supplemented with 30  $\mu\text{M}$  hypoxanthine (controls).



In the preceding paper we presented evidence that when purines are withdrawn from an exponentially growing culture of V79 pur 1 cells, cell growth is arrested and ongoing DNA synthesis is inhibited. While the uptake of  $^3\text{H}$ -thymidine incorporation into acid precipitable material rapidly decreases to 20% of the initial value, the fraction of cells incorporating thymidine in autoradiographic preparations decreases to a lesser extent (50% of the initial fraction). Further, fluoromicrofluorographic analysis shows that the cells are arrested in early S phase. Purine deprivation also causes an upset in the balance of the deoxyribonucleoside triphosphate pools, with a decrease of the purine dNTP pools and an increase of the pyrimidine dNTP pools. Supply of all four dNTPs in equal amounts in vitro leads to resumption of normal DNA synthesis.

Various reports in the literature have shown that depletion of the purine dNTP pools by use of drugs, such as hydroxyurea, inhibits the DNA chain elongation process and causes the accumulation of Okazaki fragments that cannot be incorporated into mature DNA strands. In light of the similar findings in our system presented in the preceding chapter an attempt was made to further characterize the lesion in DNA synthesis in the mutant cells and the results are presented in the following chapter, which is organized in the form of a manuscript suitable for publication.

In our analysis we use equilibrium and velocity centrifugations to characterize the newly synthesized DNA under conditions of purine deprivation. We also use DNA fiber autoradiography for a direct examination of replicating chromosomes in the purine deprived cells.

CHAPTER 3

INHIBITION OF DNA CHAIN ELONGATION IN A  
PURINE AUXOTROPHIC MUTANT OF CHINESE HAMSTER\*

\*This chapter has been submitted for publication to Cell (Maria Zannis-Hadjopoulos and Roger Hand).

### Summary

DNA synthesis has been examined in a purine auxotrophic mutant cell line of Chinese hamster (V79 pur 1) under conditions of purine deprivation. Six h after the removal of purines from the growth medium, there is a decrease in semiconservative DNA replication. During the same period, histone synthesis is also inhibited but not to the same extent. Alkaline velocity centrifugation and agarose gel electrophoresis of the DNA synthesized during a 1 min pulse under conditions of purine deprivation shows that approximately 50% of the newly replicated DNA is the size of Okazaki pieces. These are not incorporated into bulk DNA during a 1 h chase. If the purine supply is restored to the growth medium, these short DNA pieces are joined to full size DNA within 1 h. DNA-fiber autoradiography reveals a retardation in the rate of DNA replication fork movement but no apparent inhibition of initiation of synthesis on replication units within clusters actively engaged in replication. Our results indicate that purine deprivation specifically inhibits elongation of nascent DNA chains.

### Introduction

DNA synthesis is an elaborately regulated process whose many steps are not completely understood yet. One of the basic requirements for DNA replication is the continuous supply of the four deoxyribonucleoside triphosphates (dNTPs) that serve as building blocks. These are also thought to play a regulatory role in the replication process (Reichard, 1978). Numerous studies have shown that treatment of cells with hydroxyurea, which depletes the intracellular purine dNTP pools (Walters, Tobey and Ratliff, 1973; Skoog and Bjursell, 1974), inhibits the DNA chain elongation process and causes the accumulation of Okazaki fragments that cannot be incorporated into mature DNA strands (Magnusson et al., 1973; Magnusson 1973a, b; Walters, Tobey and Hildebrand, 1976a). We have in our hands a purine auxotrophic mutant cell line of Chinese hamster, designated as V79 pur 1, which is deficient in phosphoribosylpyrophosphate amidotransferase (E.C.2.4.2.14), the first enzyme of the de novo purine biosynthetic pathway (Feldman and Taylor, 1975). In another study we have shown that when V79 pur 1 cells are deprived of purines, the size of the purine dNTP pools decreases while that of the pyrimidine dNTP pools increases. The pools that are affected the most are dATP, which decreases 3-fold, and dCTP, which increases 2-fold following purine withdrawal from the growth medium. At the same time DNA synthesis is markedly reduced and the cells become arrested in the S phase (Zannis-Hadjopoulos, Baumann and Hand, manuscript submitted).

Knowing the biochemical defect in the V79 pur 1 cell line, and in light of our previous finding concerning the alterations in the dNTP pools in this mutant, we have undertaken a study to characterize the precise step in DNA replication that is affected when the supply of

purines is restricted. In this paper we present evidence that purine deprivation in these mutants delays the maturation of newly synthesized Okazaki pieces to full size DNA and also inhibits the rate of DNA replication fork movement.

### Experimental Procedures

#### Cells and Experimental Conditions

The Chinese hamster line, V79 pur 1, was used. This cell line is auxotrophic for purines and has a mutation in the gene for phosphoribosyl pyrophosphate amido transferase (E.C.2.4.2.14; Feldman and Taylor, 1975). It was maintained in our laboratory in Hamm's F10 medium supplemented with 5% fetal calf serum, amphotericin B (0.25  $\mu\text{g/ml}$ ), gentamicin (50  $\mu\text{g/ml}$ ) and tylocine (60  $\mu\text{g/ml}$ ). The details of the experimental conditions have been previously described (Zannis-Hadjopoulos et al., manuscript submitted). In brief, the day before the experiment the cells were transferred to plastic petri dishes at a density of  $2 \times 10^5$  cells per 60 mm dish and allowed to attach and grow overnight in F10 medium supplemented with 5% FCS. In experimental cultures, the cells were depleted of purines and placed under restrictive growth conditions by replacement of the growth medium with minimal essential medium (MEM) supplemented with 5% dialysed FCS. Control cultures received the same medium that had been supplemented with 30  $\mu\text{M}$  hypoxanthine.

#### Density Labeling and Extraction of DNA

Cell cultures that had been deprived of purines for 2 h were incubated in medium containing 5'-fluorodeoxyuridine (FdUrd;  $2 \times 10^{-6}$  M), bromodeoxyuridine (BrdUrd;  $10^{-5}$  M) and  $^3\text{H}$ -thymidine ( $10^{-7}$  M; 10  $\mu\text{Ci/ml}$ ) for a



period of 8 h in the dark. Parallel control cultures were identically treated. At the end of the incubation period each of the cultures was mixed with an equal number of cells that had been labelled for 16 h with  $^{14}\text{C}$ -thymidine and the DNA was extracted as described by Britten et al. (1974). The extracted DNA was dissolved in TNE (0.05 M Tris, pH 8.0, 0.15 M NaCl, 0.05 M EDTA) and sheared to a size of  $8\text{-}9 \times 10^6$  daltons by passing it four times through a 25-gauge needle.

#### Isopycnic Centrifugation in Neutral CsCl Gradients

An aliquot of about 3  $\mu\text{g}$  of the extracted DNA was brought to a final volume of 5 ml in a solution containing TNE and CsCl (density 1.72 g/ml). Centrifugation was at 33,000 rpm for 60 h at  $18^\circ\text{C}$  in an S40.2 rotor in an L2-65B Beckman ultracentrifuge. Fractions of 0.2 ml were collected from the bottom by displacement using a constant volume fractionator (M.R.A. Corporation, Boston, Mass.). The refractive index of every fifth fraction was recorded and the radioactivity in each fraction was measured by liquid scintillation counting.

#### Extraction and Analysis of Histones

Cells were labelled with  $^{14}\text{C}$ -lysine ( $3.2 \times 10^{-4}$  M; 0.5  $\mu\text{Ci/ml}$ ) for 48 h in Ham's F10 medium. The radioactive medium was replaced by purine-deprived medium containing one tenth the normal concentration of lysine. After 4 h, half the cultures received purine-deprived medium containing  $^3\text{H}$ -lysine ( $3.7 \times 10^{-4}$  M; 25  $\mu\text{Ci/ml}$ ) while the other half received the same medium containing in addition hypoxanthine (30  $\mu\text{M}$ ) to serve as control. After a labeling period of 2 h, acid-soluble proteins were extracted as previously described (Winocour and Robbins, 1970) and the extracts were subjected to electrophoresis on 10% polyacrymide gels (Hand and Kasupski, 1978).

### Velocity Sedimentation in Alkaline Sucrose Gradients

Samples containing approximately  $10^5$  cells were slowly pipetted into 0.3 ml of 0.2 M NaOH - 0.001 M EDTA, layered over a gradient of 5 - 20% sucrose in 0.9 M NaCl, 0.3 M NaOH, and 0.001 M EDTA resting on a cushion of 70% sucrose (Sheinin, 1976). Cell lysis and DNA denaturation were allowed to proceed at 4°C for 8-12 h. The gradients were then centrifuged for 16 h at 26,000 rpm at 4°C in an SW 50.1 rotor in an L2-65 B Beckman ultracentrifuge. Fractions of 0.2 ml each were collected from the bottom and counted for radioactivity.  $^3\text{H}$ -labeled  $\phi\text{X174}$  DNA (form II; from Dr. D. Denhardt, McGill University) was used as a marker.

### Agarose Gel Electrophoresis

Horizontal slab agarose gels were used as described by McDonell, Simon and Studier (1977). The gels were prepared and run in a neutral electrophoresis buffer (0.04 Tris, pH 7.8, 0.005 M sodium acetate, 0.001 M EDTA). Samples containing approximately  $10^5$  cells were lysed in 0.5 M NaCl, 0.015 M sodium citrate, 1% sarcosyl for 30 min at room temperature and the lysates were lyophilized and then dissolved in a small volume of the electrophoresis buffer. They were then heated to 100°C for 5 min and transferred into the sample wells of a 0.7% agarose gel using a micropipet. Bromophenol blue (BPB) was placed in a separate well to allow monitoring of the run. Electrophoresis was carried out at 12 mAmps until the BPB marker had migrated to a distance of approximately 5 cm (3.5 - 4h). The gels were then treated with 10% TCA for 30 min and sliced into 1 mm sections. The slices were placed into scintillation vials and autoclaved for 2 min to dissolve the agarose. They were subsequently analysed for radioactivity by scintillation counting in Aquasol (New England Nuclear).

### DNA Fiber Autoradiography

The method of Hand and Tamm (1973) was used. In brief, at times after exposure to purine-deprived or purine-supplemented medium, the cells were subjected to sequential 30 min pulses with  $^3\text{H}$ -thymidine, first at high specific activity ( $5 \times 10^{-6}$  M; 50 Ci/mmol) and then at low specific activity ( $5.5 \times 10^{-5}$  M; 5 Ci/mmol). The cells were exposed to FdUrd ( $2 \times 10^{-6}$  M) from 30 min before the pulse to its completion. At the end of the radioactive pulses, a drop of cell suspension (about  $2-3 \times 10^3$  cells) was lysed gently on a subbed glass microscope slide with a drop of a lysis buffer (PBS) containing sodium dodecyl sulfate (1%) and EDTA (0.01 M). The DNA fibers released from the cells were spread over the surface of the slide with a glass rod, air-dried, fixed, and processed for autoradiography. Exposure time was 4 - 6 months. All autoradiograms were examined using a light microscope equipped with a frosted-glass screen. The autoradiographic figures were projected on the screen and traced on clear acetate.

### Results

DNA synthesis, as measured by  $^3\text{H}$ -thymidine incorporation into acid-insoluble material is reduced to less than 10% of controls when V79 pur 1 cells are deprived of purines for up to 8 h (Feldman and Taylor, 1975; Zannis-Hadjopoulos et al., manuscript submitted).

### Semiconservative Replication

Equilibrium density centrifugation in neutral CsCl gradients was used to investigate the type of DNA synthesis performed by the mutant cells in the purine-deprived medium. A replicative type of synthesis would be expected to result in double helices with a buoyant density higher than

normal due to the incorporation of BrdUrd into the nascent strands. Repair synthesis would not cause a shift since BrdUrd would be incorporated into regions too short to alter the density of the repaired stretches of DNA. Fig. 1 shows that there is no difference in the gradient profiles of the DNA extracted from cells which were either purine-deprived or purine-supplemented (control) for 10 h. DNA synthesized in the presence of BrdUrd and  $^3\text{H}$ -thymidine banded at a density of 1.75 g/ml, characteristic of the hybrid product of semiconservative replication (Flamm, Bond and Burr, 1966) whereas the normal unsubstituted marker DNA banded at a density of 1.70 g/ml. The bulk of the  $^3\text{H}$ -label is associated with the high-density fractions rather than with DNA of normal density. This result indicates that the residual DNA synthesized in the mutant cells deprived of purines is made in semiconservative fashion characteristic of the S phase. It also indicates that there is little or no repair replication and no breakdown of preexisting DNA strands.

### Histone Synthesis

The relationship of histone synthesis to DNA synthesis and the cell cycle has been extensively investigated in a variety of cell systems (reviewed by Elgin and Weintraub, 1975; Prescott, 1976). It has been observed in many mammalian cells that the synthesis of histones is generally tightly coupled to that of DNA (Edenberg and Huberman, 1975; Elgin and Weintraub, 1975) but a few cases of uncoupling of the two processes have been reported, such as in G<sub>1</sub>- or early S-arrested cells (Gurley and Hardin, 1968; Gurley, Walters and Tobey, 1972; Prescott, 1976; Raydt et al. 1977) and in SV40-infected cells in which host DNA synthesis is inhibited (Liberti, Fischer-Fantuzzi and Vesco, 1976). It seemed of interest to

analyse the effect of purine deprivation on the synthesis of histones in the V79 pur 1 mutants, at a time when DNA synthesis is inhibited and the cells are arrested in the S phase.

Acid-soluble nuclear proteins were extracted from purine-depleted and purine-supplemented cells that had been labeled with  $^{14}\text{C}$ -lysine before depletion and  $^3\text{H}$ -lysine 4-6 h after depletion. The extracts were analysed by electrophoresis on 10% polyacrylamide gels. The electrophoresis profiles are shown in Fig. 2. Two major lysine rich peaks that contain the five classes of histones (Winocour and Robbins, 1970) were resolved on the gels. The amount of  $^3\text{H}$ -lysine incorporated into the histone peaks of cells deprived of purines (Fig. 2a) is about 50-60% of that incorporated into the control cells (Fig. 2b) as determined by the ratios of  $^3\text{H}:^{14}\text{C}$  incorporated into those peaks. Overall DNA synthesis during the same time interval was estimated from the ratio of  $^3\text{H}:^{14}\text{C}$  in acid-insoluble material from cells labeled for 16 h with  $^{14}\text{C}$ -thymidine before being exposed to purine-deprived medium for 6 h and then labeled with  $^3\text{H}$ -thymidine for 15 min. The results of these experiments and the histone synthesis experiments are compared in Table 1. The inhibition of DNA synthesis is more marked than that of histones, indicating an uncoupling of the two processes in the purine-deprived cells. This result conforms with our previous finding that purine deprivation causes an immediate arrest of the cells in the S phase (Zannis-Hadjopoulos et al., manuscript submitted) and is in agreement with the results obtained by other investigators, where relatively high rates of histone synthesis were detected in the absence of DNA synthesis in cells that were arrested early in the S phase (Gurley and Hardin, 1968; Gurley et al., 1972).

### Characterization of the newly-synthesized DNA

Once we established that DNA synthesis was replicative in the absence of purines, we designed pulse-chase experiments to determine the size of nascent DNA strands under purine-depleted conditions. V79 pur 1 cells whose bulk DNA had been prelabeled with  $^{14}\text{C}$ -thymidine were incubated in purine-deprived or purine-supplemented medium for 6 h. At this time approximately 50% of the cells have entered S, as determined by flow microfluorography, but only about 30% of the cells are actively engaged in DNA synthesis, as determined by autoradiography (Zannis-Hadjopoulos et al., manuscript submitted).  $^3\text{H}$ -thymidine ( $2 \times 10^{-6}$  M; 50 Ci/mmole) was added to control and depleted cells for 1 min to pulse-label the nascent DNA. The cells were then processed immediately for analysis on alkaline sucrose gradients and on agarose gels, or incubated for an additional hour in the appropriate medium containing non-radioactive thymidine in excess ( $2 \times 10^{-5}$  M).

Typical gradient patterns are shown in Fig. 3. The distribution of radioactivity is the same in the purine-deprived and purine-supplemented cells after 1 min of pulse-labeling with  $^3\text{H}$ -thymidine (Fig. 3a and c). In both, approximately 50% of the  $^3\text{H}$ -labeled DNA is recovered as small molecular weight material that sediments at 4 to 5 S near the top of the gradients, while the remainder cosediments with full-sized  $^{14}\text{C}$ -prelabeled DNA ( $\geq 46\text{S}$ ). In the pulse-chase experiments, however, a chase period of one hour was not enough to allow conversion of the low molecular weight molecules into bulk-size DNA in the purine depleted cells (Fig. 3b) whereas in control cells, all the DNA was incorporated into full-sized molecules within this time (Fig. 3d). Although apparently some low molecular weight material was chased into longer strands in one hour in

the purine-deprived cells, 50% of the radioactivity recovered as 4-5s material after the 1 min pulse remained as such after a 1 h chase.

If the cells were deprived of purines for 6 h and were subsequently exposed to medium supplemented with hypoxanthine, the ability to chase the low molecular weight DNA into bulk-size was regained within 1 h (Fig. 3e and f). This ability was also present after 23 h of purine-deprivation (Fig. 1 Appendix) when repletion of the purine ribonucleotide (Sriram and Taylor, 1977) and deoxyribonucleotide pools (Zannis-Hadjopoulos et al, manuscript submitted) occurs due to breakdown in ribosomal RNA (Sriram and Taylor, 1977). These results suggest that in the absence of purines there is a retardation in the conversion of small Okazaki-type pieces of newly replicated DNA to large molecular weight DNA.

Identical results in depleted cells were obtained when the samples were subjected to electrophoresis on agarose gels, as shown in Fig. 4. After 1 min of pulse-labeling with  $^3\text{H}$ -thymidine, two major size classes of DNA are resolved on the gels, one comigrating with the bulk-size  $^{14}\text{C}$ -prelabeled DNA and the other migrating as a peak around  $9.5 \times 10^5$  daltons, ahead of the  $\phi\text{X174}$  marker (Fig. 4a and c). This peak can be chased into bulk-size DNA within 1 h in purine-supplemented cells (Fig. 4d). In purine-deprived cells after 1 h chase there is still a prominent peak of small molecular weight material but there is also a broader distribution of intermediate size material of  $1-5.5 \times 10^6$  daltons (Fig. 4b). This material might represent replication intermediates similar to those observed in other systems (Tseng and Goulian, 1975), detectable only in the purine-deprived cells after 1 h of chase because of retardation in the formation of mature DNA strands. It is unlikely that they

represent breakdown material of larger DNA as indicated by the distribution profile of the  $^{14}\text{C}$ -prelabeled bulk-size DNA, which migrates as a homogenous peak.

#### Rate of DNA Replication Fork Movement

As a result of the above findings, which suggest a defect in the process of elongation, we decided to look directly at the effect of purine depletion on the rate of DNA replication fork movement in V79 pur 1 cells by means of DNA fiber autoradiography. The rate was determined by measuring the length of the high-grain-density tracks of DNA generated during the high-specific activity pulse of  $^3\text{H}$ -thymidine on units that initiated synthesis before the pulse, and then dividing by the duration of the pulse (Hand and Tamm 1973). Track lengths from control and depleted cells are approximately normally distributed at all time points. The mean track length values obtained as well as the calculated rates are shown in Table 2. The rates in purine-supplemented cells are similar to those previously found for Chinese hamster cells (Sheinin, Humbert and Pearlman, 1978; Edenberg and Huberman, 1975; Yurov and Liapunova, 1977). Within 4 h in purine-depleted medium, the rate of fork movement declines to 58% of the control rate and by 6 h reaches 36% of the corresponding rate found in the purine-supplemented cells. In cells deprived of purines for 4 h and then switched to purine-supplemented medium for 2 h, the rate rises again to 92% of the corresponding control. Representative autoradiograms of 6 h purine-deprived and purine-supplemented cultures are shown in Fig. 5, in which the difference in the track lengths between the two is clearly visible. The above results indicate that purine deprivation has a direct inhibitory effect on the rate of DNA replication fork movement.



### Interval Between Initiation Sites

If initiation were inhibited in addition to fork movement, we might expect to see other alterations in the patterns of fiber autoradiograms in the depleted cells. Among these could be an increase in the interval between initiation sites caused by a block to initiation at some sites that would be used in the normal course of replication in non-depleted cells.

The interval between active sites was determined by measuring the center-to-center (intertrack) distances between adjacent replication units. The method of selection of autoradiograms for the scoring of intervals was as described in Hand and Tamm (1974). In brief the autoradiograms are collected from areas of the slide where the DNA is well spread, and two units are considered to be on the same fiber if they are aligned and within the same microscopic field at a magnification of 280. With this method, neighboring units that lie far apart are overlooked because of the difficulty in recognizing their alignment and, as a consequence, the results tend to be biased toward smaller intervals, as it has also been pointed out previously (Huberman and Riggs, 1968; Callan, 1973; Hand and Tamm, 1974; Martin and Oppenheim, 1977). The mean intertrack distances are shown in Table 3. They are expressed as geometric means rather than arithmetic means because the distributions of such distances are not Gaussian but rather skewed toward larger values, as it has also been observed previously by other investigators (Blumenthal, Kriegstein and Hogness, 1973; Hand and Tamm, 1974; Hand, 1977; Hand and German, 1977; Martin and Oppenheim, 1977). The geometric mean is preferred because of its "weighting" characteristics in the statistical sense, whereby it gives smaller observations relatively more importance than

does the arithmetic mean (Martin and Oppenheim, 1977; Oppenheim and Martin, 1978). It can be seen that intertrack distances become progressively smaller with increasing time in the purine-deprived medium. The intertrack distances observed in the control cells are within the range previously reported for Chinese hamster cells (Huberman and Riggs, 1968; Yurov and Liapunova, 1977). Cumulative frequency distributions of intertrack distances are presented in Fig. 6. At all time-points considered, the purine-deprived cells have the highest frequencies of short intertrack distances, as indicated by the initial steep slopes in their cumulative frequency curves. The median values are 76-88  $\mu\text{m}$ . The cumulative frequency curves of the purine-supplemented cells indicate a much broader distribution of intertrack distances with median values of 108-112  $\mu\text{m}$ . Thus, purine-deprivation is not associated with an increase in initiation intervals. This suggests that initiation on active clusters of replication units is not inhibited by purine depletion.

#### Relative Frequency of Initiation

As an additional test of whether initiation is inhibited by purine depletion, we measured the frequency of initiations by scoring the fraction of replication units that had initiated before the high-specific activity pulse (prepulse units) and comparing it to the fraction of units that initiated during the high-specific activity pulse (postpulse units), as described by Hand (1975b). A decrease in the frequency of initiations would result in a decrease in the fraction of postpulse initiation units relative to controls (purine-supplemented cells). Scoring was done blind on coded slides; the results are shown in Table 4. Purine-deprived cells show the same proportion of postpulse units as the purine-supplemented

cells at all time points examined. We conclude, therefore, that purine-deprivation does not inhibit initiation of replication units on active clusters.

### Discussion

Our results show that under conditions of purine deprivation, DNA synthesis in V79 pur 1 cells is inhibited. Residual synthesis is replicative in nature, a characteristic of S phase synthesis, and histone synthesis is reduced to a lesser extent than overall DNA synthesis. Depleted cells lose the ability to convert small, newly replicated DNA strands (Okazaki pieces) to mature-size chromosomal DNA. This is associated with a marked reduction in the rate of DNA replication fork movement. Purine deprivation has no apparent effect on the frequency of initiation of new DNA synthesis. All inhibitory effects observed under conditions of purine deprivation are reversible; restoration of purines into the medium results in resumption of normal DNA synthesis.

These results show that purine deprivation inhibits semiconservative DNA synthesis by directly inhibiting the rate of DNA chain elongation, as measured by replication fork progression and maturation to full size DNA. A block in maturation has been demonstrated by other investigators in systems in which a state of purine depletion was induced through treatment with hydroxyurea (Magnusson et al., 1973; Magnusson 1973 a, b; Walters et al., 1976 a,b). Purine-deprived V79 pur 1 cells are arrested in S, as we have shown (Zannis-Hadjopoulos et al, manuscript submitted) and this is also true for hydroxyurea-treated cells (Walters et al, 1976 b). Although dATP availability may be a major rate limiting factor for DNA synthesis (Zannis-Hadjopoulos et al., manuscript submitted), we feel that a balance between all four deoxyribonucleoside triphosphate pools must exist in the cells for normal DNA synthesis to occur.

The possibility that the small-size DNA persisting under purine-deprived conditions is a breakdown product of pre-existing DNA strands can be excluded for 2 reasons: a) In alkaline sucrose gradients as well as in agarose gels, the  $^{14}\text{C}$ -prelabeled DNA marker is recovered always as one peak at a position characteristic of bulk DNA, and b) the small DNA could be chased into bulk DNA immediately upon repletion of the deprived medium with purines. We cannot exclude the possibility that these small DNA fragments may not represent true Okazaki pieces, but may be instead the result of an excision repair process arising by misincorporation of uracil into DNA and subsequent removal, similar to that operating in bacteria (Tye and Lehman, 1977) and mammalian systems (Sekiguchi et al., 1976; Brynolf, Eliasson and Reichard, 1978; Grafstrom, Tseng and Goulian, 1978). Misincorporation of uracil into DNA is more frequently observed when dUTP levels are elevated (Tye and Lehman, 1977). In mammalian cells, dUTP is synthesized by phosphorylation of dUMP that is formed by deamination of dCMP (Maley and Maley, 1959). Although, normally dUTP would be converted rapidly back to dUMP (Bertani, Häggmark and Reichard, 1963) and then to dTTP, this reaction may be inhibited to some extent in purine-deprived V79 pur 1 cells because of the increased size of the dCTP and dTTP pools.

Against the likelihood of repair synthesis during purine deprivation are the following observations: a) in the absence of purines all of the  $^3\text{H}$ -thymidine is incorporated along with BrdUrd into molecules of hybrid density, and b) during a half-hour pulse-labeling period  $^3\text{H}$ -radioactivity is incorporated into discrete stretches of DNA measurable by DNA fiber autoradiography rather than into small areas dispersed randomly throughout the DNA. However, the possibility remains that some of the small DNA molecules seen in depleted cells arise from excision of replicating stretches into which uracil was misincorporated.

The overall rate of DNA synthesis is determined by two factors, the rate of replication fork movement and the frequency of initiation of replication units. Our results indicate that replication fork movement is inhibited by purine depletion, but we cannot provide evidence that initiation is blocked. However, fiber autoradiography permits examination only of those clusters of replication units active at the time of the radioactive pulse. Since depleted cells are arrested in S phase (Zannis-Hadjopoulos et al., manuscript submitted), initiation must be inhibited at some point, probably on whole clusters of replication units. We are unable to detect this by fiber autoradiography.

We also observed a decrease in the interval between initiation sites in the purine-depleted cells in comparison to their nondepleted counterparts. The correlation of reduced rate of replication fork movement and smaller intertrack distance has also been observed by other investigators (Taylor and Hozier, 1976; Ockey and Saffhill, 1976; Kurek and Taylor, 1977; Taylor, 1977). A decrease in the intertrack distance may reflect an increase in the number of active initiation sites operating along the DNA fiber (Taylor and Hozier, 1976; Martin and Oppenheim, 1977; Oppenheim and Martin, 1978). Our results, however, show that the frequency of initiations in the purine-deprived cells does not differ from that in the purine-supplemented cells, despite the fact that intertrack distances are significantly smaller in the former. We have no clear explanation for the reduced intervals in depleted cells. The longer intervals in the control cells cannot be accounted for by fusion of strands from adjacent units that completed synthesis before the end of the pulse, since the rate of fork movement is slow enough to permit operation of almost all replication units for longer than the 30 min pulse of high specific

activity. It is possible, however, that some fusion does take place in units replicating at a slightly faster rate than the average. A correlation between size of replication units (and therefore initiation intervals) and the rate of replication fork movement has been observed (Ockey and Saffhill, 1976). It is possible that in clusters of small replication units, the replication forks are retarded by constraints imposed by the relatively close proximity of the stretches of the DNA molecule undergoing replication. If this is so, then one explanation for our findings is that initiation on clusters of large replication units is selectively inhibited by purine depletion and residual synthesis on the clusters of small units has a slower rate of fork movement. Even if this is correct, there must be an additional direct effect of purine depletion on synthesis to account for the marked inhibition in elongation and the accumulation of small DNA molecules. Thus, an adequate supply of purines seem to be required for polymerization, gap-filling and ligation in mammalian DNA synthesis.

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Acknowledgements

We thank Dr. M.W. Taylor for providing us with the V79 pur 1 cells and for his helpful ideas and suggestions in the designing of some of the experiments. This work was supported by grants from the Medical Research Council of Canada and the National Cancer Institute of Canada and has been in partial fulfilment of the requirements for the Ph.D. degree from McGill University for M.Z.-H.

Table 1 - Histone and DNA Synthesis in Purine-deprived and Purine-Supplemented (control) V79 pur 1 Cells<sup>a</sup>

Radioactivity	Histone Synthesis				DNA Synthesis	
	Peak 1 <sup>b</sup>		Peak 2 <sup>b</sup>		Depleted	Control
	Depleted	Control	Depleted	Control		
<sup>3</sup> H (cpm)	3,912	6,370	10,880	22,419	6,785	33,087
<sup>14</sup> C (cpm)	2,204	2,267	5,398	5,497	1,340	1,466
<sup>3</sup> H: <sup>14</sup> C <sup>c</sup>	1.7 (60)	2.8	2.0 (48)	4.1	5.1 (23)	22.6

<sup>a</sup>Cells were either deprived or supplemented with purines for 6 h.

<sup>b</sup>Peak 1 represents fractions 65-75 and peak 2 represents fractions 76-85 from the gels in Figure 2.

<sup>c</sup>The <sup>3</sup>H:<sup>14</sup>C ratio was calculated for histones from incorporation of radioactive lysine into the histone peaks of the polyacrylamide gel, and for DNA from incorporation of radioactive thymidine into acid-insoluble material. For DNA synthesis, the values after 6 h of purine depletion are shown. An identical degree of inhibition was observed after 4 h of depletion. Values in parenthesis show the percent of histone or DNA synthesis in purine-deprived cells compared to controls.

Table 2 - Replication Fork Movement in Purine-Deprived and Purine-Supplemented V79 pur 1 Cells

Time (h)	Medium	Track Length $\bar{X} \pm$ S.D. ( $\mu\text{m}$ )	Rate ( $\mu\text{m}/\text{min}$ )	% of control	No. of tracks measured
0		39.2 $\pm$ 2.87	1.3	-	198
4	Control <sup>a</sup>	37.2 $\pm$ 3.71	1.2	-	200
	Depleted	22.2 $\pm$ 1.72	0.7 <sup>b</sup>	58	202
	Depleted/repleted for 2 h	32.4 $\pm$ 3.67	1.1	92	200
6	Control	42.8 $\pm$ 3.78	1.4	-	200
	Depleted	15.1 $\pm$ 1.33	0.5 <sup>b</sup>	36	200

<sup>a</sup> Medium supplemented with 30  $\mu\text{M}$  hypoxanthine

<sup>b</sup> Values that are significantly different from the controls ( $P < 0.001$ ).

Table 3 - Replication Unit Lengths for Purine-Deprived and Purine-Supplemented V79 Pur 1 Cells.

Time (h)	Medium	Geometric Mean Intertrack distance ( $\mu\text{m}$ )	No. of Intertrack Distances Measured
0		110.3 $\pm$ 1.65	100
4	Control <sup>a</sup>	111.9 $\pm$ 1.64	102
	Depleted	91.2 $\pm$ 1.64 <sup>b</sup>	107
	Depleted/repleted for 2 h	97.2 $\pm$ 1.68	113
6	Control	110.4 $\pm$ 1.81	130
	Depleted	76.9 $\pm$ 1.69 <sup>c</sup>	100

<sup>a</sup> Medium supplemented with 30  $\mu\text{M}$  hypoxanthine.

<sup>b</sup> Value significantly different from the control ( $P < 0.01$ ).

<sup>c</sup> Value significantly different from the control ( $P < 0.001$ ).

Table 4 - Frequency of Initiation in Purine-Deprived and Purine-Supplemented V79 Pur 1 Cells.

Time (h)	Medium	Initiation Units Postpulse (proportion)	No. of units scored
0	Control <sup>a</sup>	0.52	326
	Depleted	0.50	430
4	Control	0.52	349
	Depleted	0.51	334
	Depleted/repleted for 2h	0.52	349
6	Control	0.52	373
	Depleted	0.53	341

<sup>a</sup> Medium supplemented with 30  $\mu$ M hypoxanthine.



Figure 1. CsCl isopycnic centrifugation of DNA synthesized by purine-deprived (a) and purine-supplemented (b) V79 pur 1 cells. Cells previously deprived of purines for 2 h were incubated in medium depleted of purines containing FdURd ( $2 \times 10^{-6}$  M), BrdUrd ( $10^{-5}$  M), and  $^3\text{H}$ -thymidine ( $10^{-7}$  M) and from which purines were omitted for an additional 8 h. Parallel purine-supplemented (control) cells were identically treated. After the labeling period, the cultures were mixed with cells pre-labeled with  $^{14}\text{C}$ -thymidine, the DNA was extracted, and an equal number of  $^3\text{H}$ -counts were loaded onto the gradients and centrifuged to equilibrium. ( $\bullet$ ),  $^3\text{H}$ -thymidine/BrdUrd-labeled DNA; ( $\circ$ ),  $^{14}\text{C}$ -labeled DNA; ( $\blacktriangle$ ), buoyant density (g/ml).

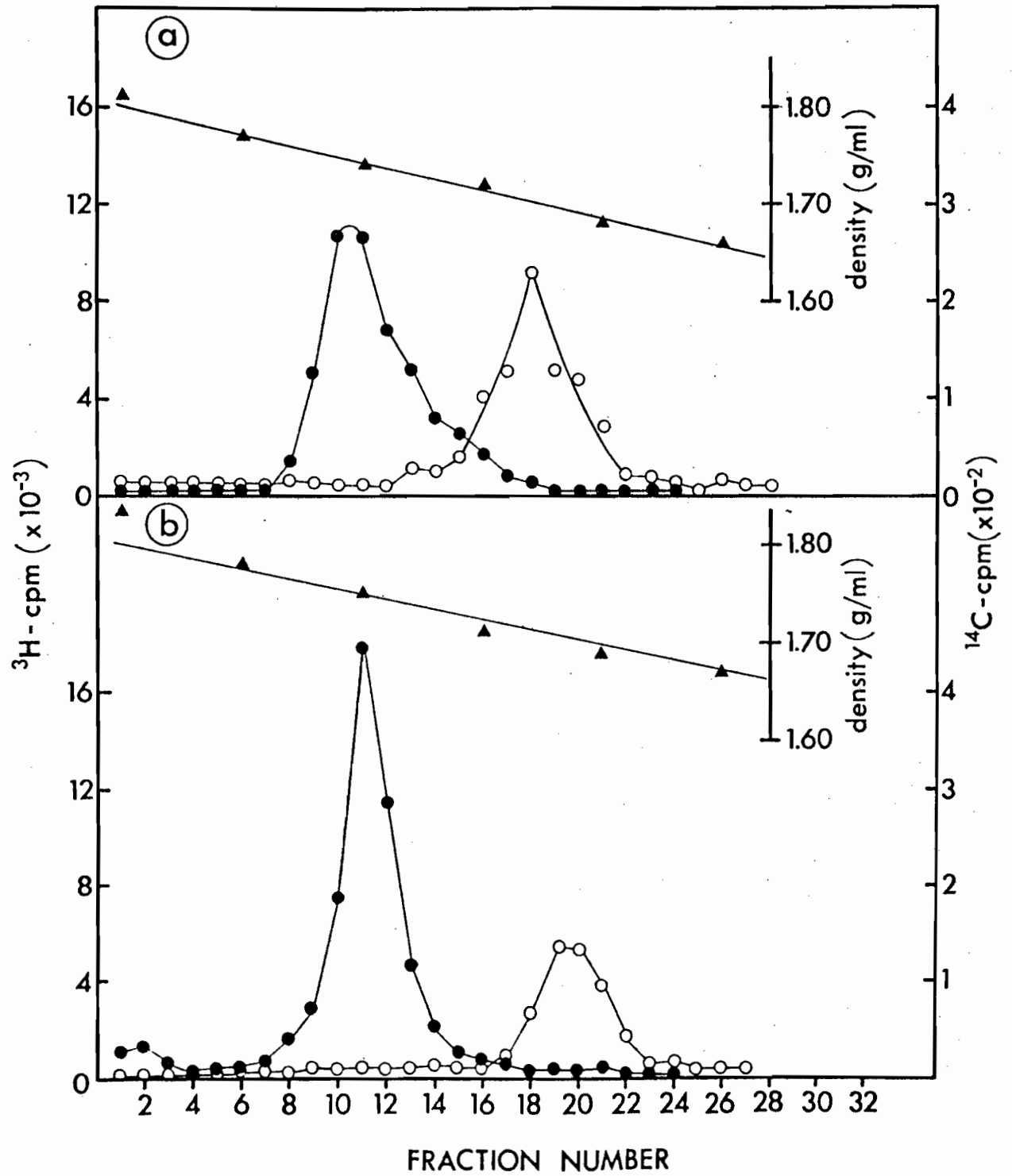


Figure 2. Effect of purine deprivation on histone synthesis in V79 pur 1 cells. Cells were pre-labeled with  $^{14}\text{C}$ -lysine ( $3.2 \times 10^{-6}$  M; 0.5 uCi/ml) for 48 h. The radioactivity was removed and the cells were incubated in purine depleted medium. After 4 h, half the cells received medium without purines containing  $^3\text{H}$ -lysine ( $3.7 \times 10^{-7}$  M; 25 uCi/ml) (a), and the other half received the same medium supplemented with 30  $\mu\text{M}$  hypoxanthine (b). After a labeling period of 2 h, acid-soluble nuclear proteins were extracted and subjected to electrophoresis on 10% polyacrylamide-SDS gels. ( $\bullet$ ),  $^3\text{H}$ -cpm; ( $\circ$ ),  $^{14}\text{C}$ -cpm.

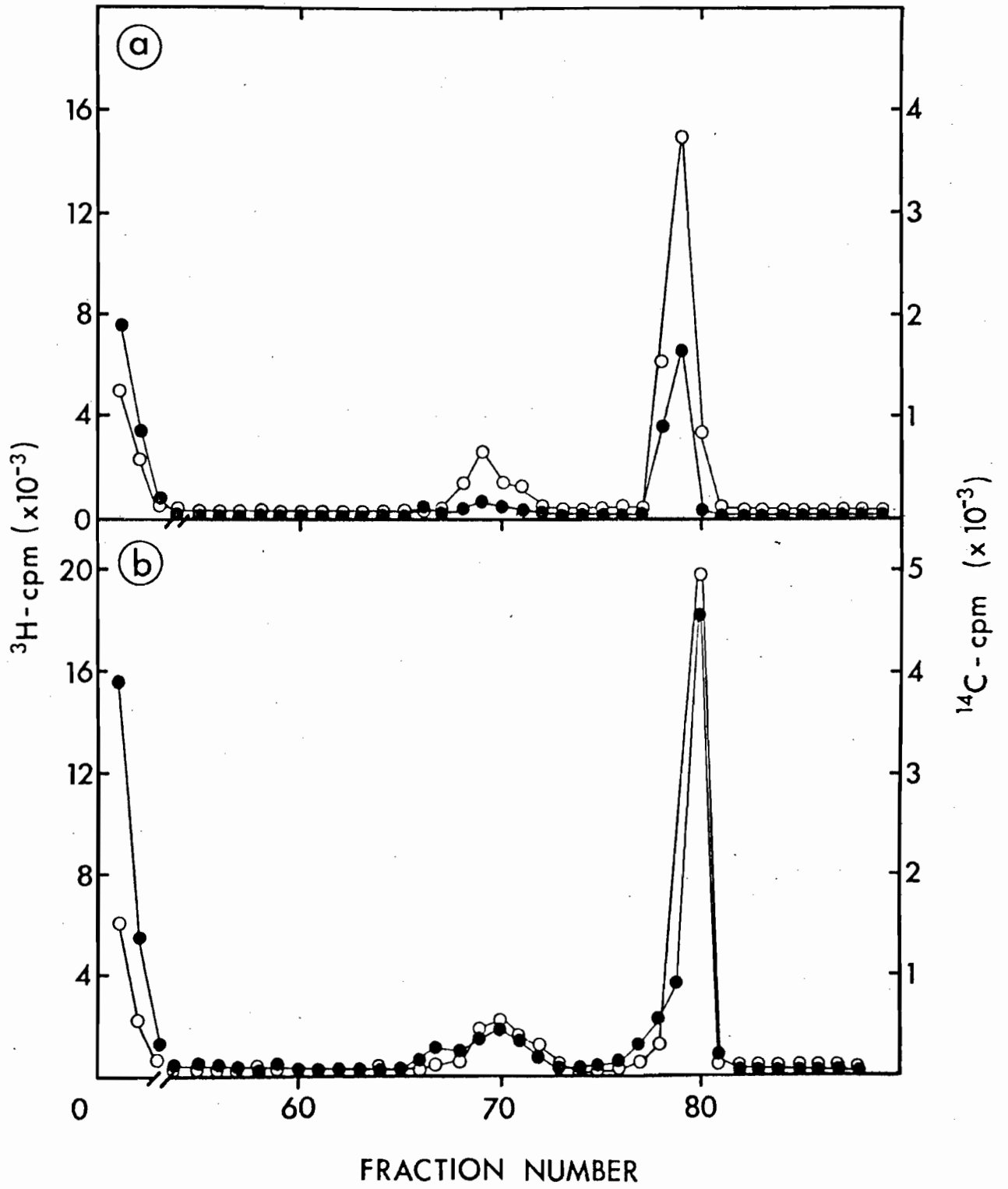
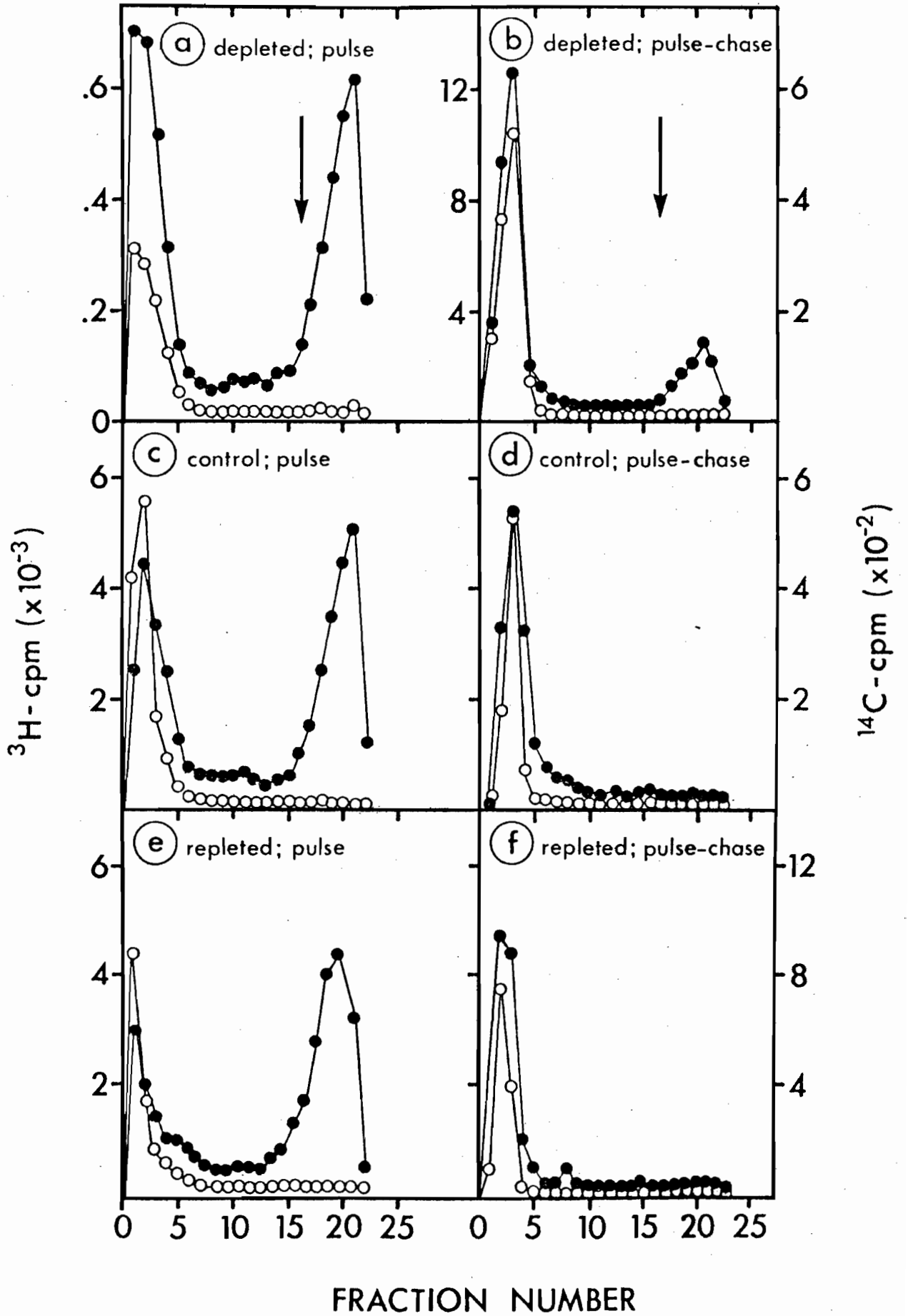


Figure 3. Alkaline sucrose gradient centrifugation of DNA synthesized by purine deprived V79 pur 1 cells in pulse-chase studies. Cells were pre-labeled with  $^{14}\text{C}$ -thymidine ( $0.01 \mu\text{Ci/ml}$ ;  $50 \text{ Ci/mmole}$ ) in F10 plus 5% FCS. After growth overnight, half the cultures received medium without purines and the other half received medium supplement with  $30 \mu\text{M}$  hypoxanthine (control). After 6 h, cells were pulse-labeled with  $^3\text{H}$ -thymidine ( $2 \times 10^{-6} \text{ M}$ ;  $50 \text{ Ci/mmole}$ ) for 1 min and were processed either immediately (panels a, c) or after 1 h chase (panels b, d) with unlabeled thymidine ( $2 \times 10^{-5} \text{ M}$ ). Some cells were deprived of purines for 4 h and then supplemented with hypoxanthine (repleted) for 1 h before the pulse (panel e) or pulse-chase (panel f) treatments. Arrow indicates position of  $\phi\text{X174}$  marker DNA run in a parallel gradient. ( $\bullet$ ),  $^3\text{H}$ -cpm; ( $\circ$ ),  $^{14}\text{C}$ -cpm.



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Figure 4. Agarose gel electrophoresis of DNA synthesized by purine-deprived V79 pur 1 cells in pulse-chase studies. Cells were prepared as in Figure 3. At the end of pulse or pulse-chase labeling, samples of purine-deprived (panels a and b) and purine-supplemented (panels c and d) cells were subjected to electrophoresis on agarose gels. Arrow indicates position of  $\phi$ X174 marker DNA run on the same gel. (•),  $^3\text{H}$ -cpm; (○),  $^{14}\text{C}$ -cpm.

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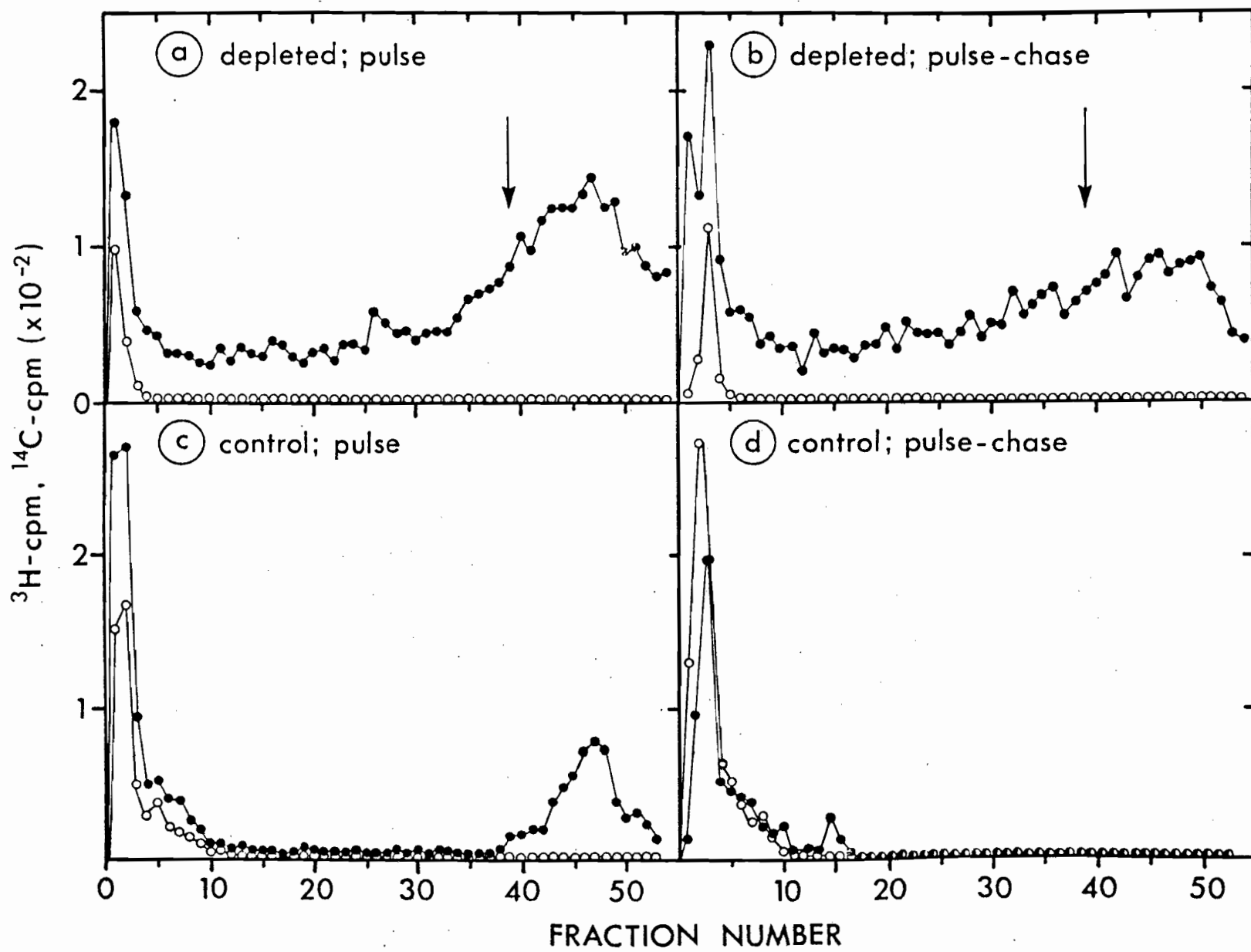




Figure 5. DNA fiber autoradiograms from V79 pur 1 cells. The cells were labeled with  $^3\text{H}$ -thymidine during a 30-min high-specific activity pulse ( $5 \times 10^{-6}$  M; 50 Ci/mmol) followed by a 30 min low-specific activity pulse ( $5.5 \times 10^{-5}$  M; 5 Ci/mmol), and were processed for fiber autoradiography. (a) purine-depleted cells; (b) purine-supplemented cells. The bar represents 50  $\mu\text{m}$ . All micrographs are at a magnification of 450.

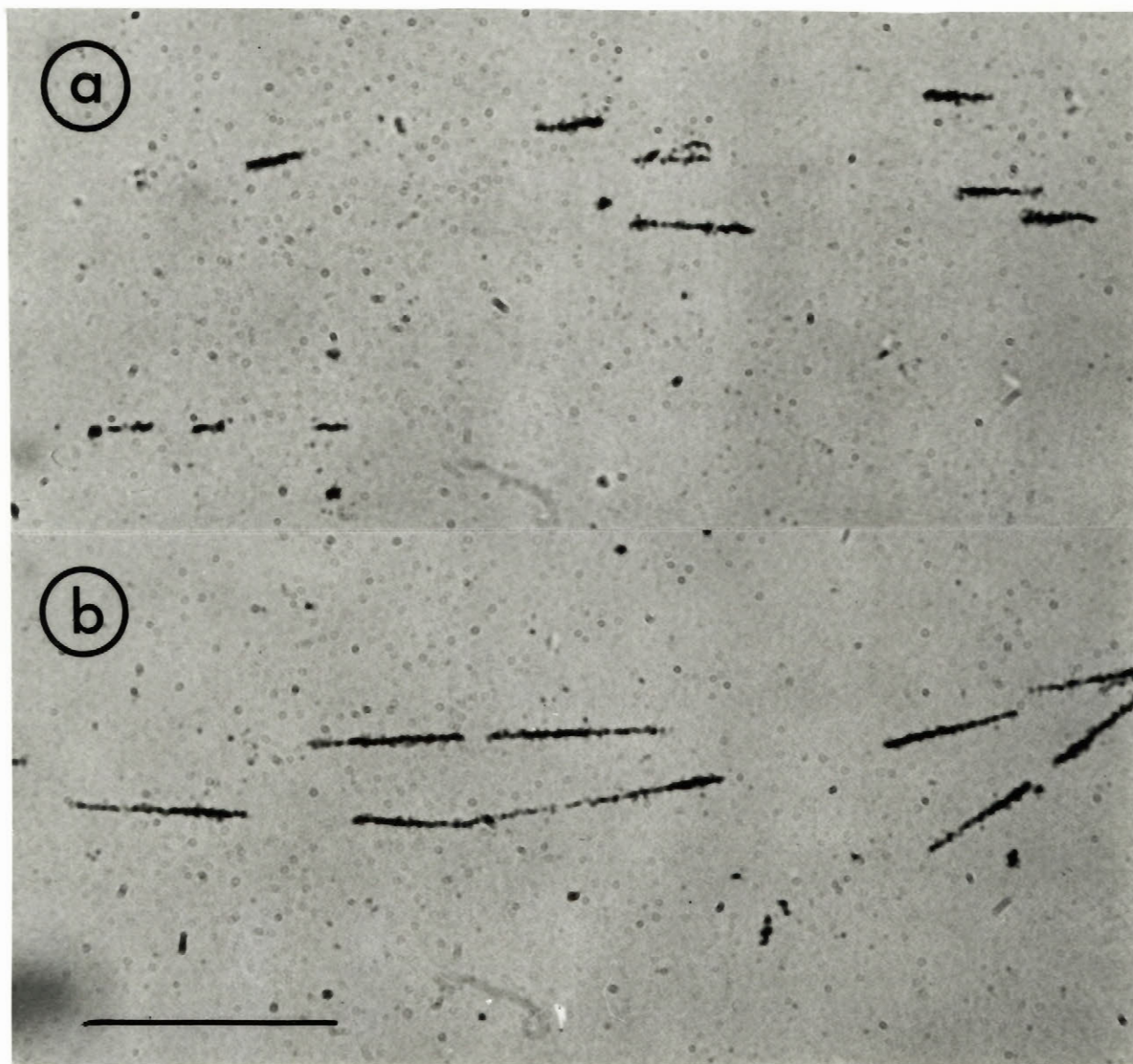
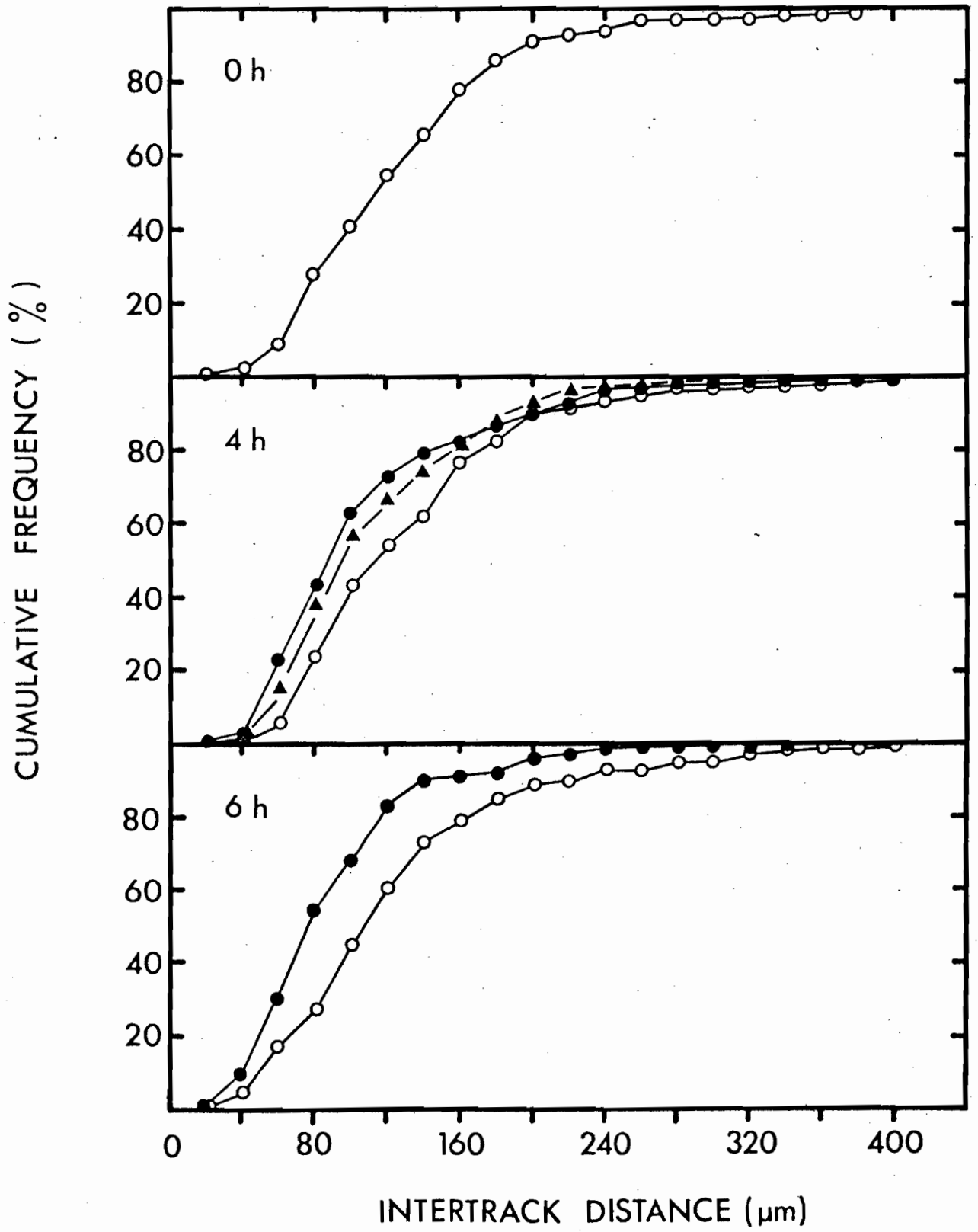


Figure 6. Cumulative frequency distributions of intertrack distances in purine-deprived and purine-supplemented V79 pur 1 cells. The number of intertrack distances measured are given in Table 3. (●), purine-deprived cells; (○), purine-supplemented cells; (▲), cells deprived of purines for 4 h and then supplemented with 30  $\mu$ M hypoxanthine for 2 h.



CHAPTER 4

GENERAL DISCUSSION

## Introduction

As mentioned in the preface, chapters 2 and 3 of this thesis have been presented in the form of manuscripts suitable for submission to learned journals for publication. As a consequence of this, the length of the discussion in the two manuscripts is restricted. It is the purpose of this chapter to provide a supplemental discussion of: 1) the scientific data presented in the above mentioned chapters, including possible alternative interpretations, and 2) the various techniques employed and their limitations.

### Limitations of the use of $^3\text{H}$ -thymidine as a monitor of DNA synthesis.

The thymidine nucleotide, dTTP, is a direct precursor to DNA. It may be supplied endogenously by conversion of dUMP to dTMP to dTTP or exogenously by the transport of thymidine across the cell membrane and its subsequent phosphorylation to dTNP and dTTP. Three factors could reduce the incorporation of exogenous thymidine into DNA other than a decrease in DNA synthesis.

1.) Transport or phosphorylation can be inhibited and cause a decrease in incorporation of radioactive thymidine into DNA with no true decrease in DNA synthesis (Lindbergh et al., 1969); 2.) An increase in de novo dTTP synthesis could cause a decrease in specific activity of the cellular dTTP pools (pool dilution); and 3.) Equilibration time of the  $^3\text{H}$ -dTTP pool could be retarded.

Although the transport of  $^3\text{H}$ -thymidine in control and purine deprived cells was not analysed, the possibility of an alteration in the transport mechanism is small since the cells under study were not treated with a toxic agent that might cause surface alterations such that might primarily affect

the transport of  $^3\text{H}$ -thymidine, as it has been reported for example in drug-treated cells (Grunicke et al., 1975; Roller et al., 1976; Hand and Tamm, 1977) and in virus-infected cells (Hand, 1976; Hand and Oblin, 1977; Hand and Kasupski, 1978; Lindbergh et al., 1969).

The mutant cell line used in the studies in this thesis was chosen specifically with the idea that a defined intracellular biochemical defect would allow the study of DNA synthesis in such a way that the primary effects on exogenous thymidine incorporation would most likely not be mediated through changes in transport.

Measurement of transport would not have aided in determining the effect of the mutation on DNA synthesis. Inhibition of DNA synthesis per se causes a secondary inhibition of thymidine phosphorylation by inhibition of thymidine kinase (Hauschka, 1973). This in turn reduces transport because of the coupling of transport and phosphorylation of nucleosides. It was awareness of this difficulty that prompted me to measure pool sizes and analyze DNA synthesis by autoradiography, since these analyses are independent of changes in thymidine transport.

The potential problem of pool dilution was handled in two ways. First, FdUrd was used as a control in my early studies on thymidine incorporation into DNA. In Figure 2 in Chapter 2, a comparison is made between the incorporation of thymidine into DNA in the presence of FdUrd and in its absence. The data show that thymidine incorporation is greater in the presence of this inhibitor of endogenous thymidine synthesis. At the concentrations used ( $2 \times 10^{-6}$  M), this compound inhibits more than 95% of the synthesis of endogenous thymidine nucleotides. The result of this is that the endogenous pool of thymidine nucleotides is virtually eliminated. Thus this experiment minimizes one of the drawbacks of measuring DNA synthesis by incorporation of exogenous thymidine, that is, the potential

confounding effect of endogenous pool dilution caused by an increase in the rate of synthesis of endogenous thymidine nucleotides. If one must rely entirely on incorporation of thymidine to measure DNA synthesis, the measurements obtained in the presence of FdUrd are probably more reliable because at least one extraneous variable has been eliminated. Second, as mentioned above, the pool measurements and fiber autoradiography results are independent of thymidine incorporation and would not be altered by pool dilution.

The problem of retarded equilibration does not enter into the interpretation of my results. Equilibration of exogenous thymidine with the cell is extremely rapid, usually less than 1 min (Richter and Hand, 1979). There are experimental situations where delays up to 5 min have been seen such as in the presence of bromodeoxyuridine (Painter and Schaeffer, 1971). Delays as long as this would not have substantially altered my results, since the  $^3\text{H}$ -thymidine pulses in the absence of bromodeoxyuridine were 15 to 30 min and the pulses in the presence of bromodeoxyuridine were several hours long. Again it is to be emphasized that the pool studies and fiber autoradiography analyses are independent of changes in equilibration time.

Comparison between  $^3\text{H}$ -thymidine incorporation into acid insoluble material and into S phase cells.

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As it can be seen in Chapter 2, Figure 2 (panels A and B, p. 66), although incorporation of  $^3\text{H}$ -thymidine into acid insoluble in the presence of FdUrd drops to 20% of the control in the purine deprived cells there is a large population of cells in S (50% of the control), as detected by autoradiography. It is these cells that I have undertaken to analyze in this study. The rest are presumably arrested in  $G_1$  and, by and large



cannot be studied by the methods employed here. It is unlikely that the cells, which were able to incorporate  $^3\text{H}$ -thymidine be leaky because they are distinctly different from their control counterparts under all aspects that were tested.

As pointed out earlier, it was felt that thymidine incorporation per se was a poor indicator of DNA synthesis and the more elaborate studies of DNA replication were undertaken to provide an accurate analysis of the effect of the mutation. It seems most likely that limiting quantities of purines produce a state in which progression through the S-phase, and therefore DNA synthesis, is immediately slowed down while cells in late  $G_1$  continue to enter S at a near normal rate. In such a case most of the purine starved cells would be in the early part of S, which, as reported by Klevecz et al. (1975) cannot be accurately determined by either total  $^3\text{H}$ -thymidine incorporation or by FMF analysis. According to that study, the most sensitive measure of DNA synthesis is autoradiography, since exposure times can be lengthened to permit detection of very low levels of net DNA synthesis, characteristics of early S. It should be noted that a similar result has been obtained in other systems in which DNA synthesis is inhibited through an S-phase block, such as in arginine deprived KB cells (Schauer-Weissfeld and Rouse, 1977) and in the temperature sensitive tNB-2 cells at the restrictive temperature (Eilen et al., personal communication). Again it should be emphasized that incorporation of  $^3\text{H}$ -thymidine in absence of FdUrd (as measured in Fig. 2A, p.86) is the least sophisticated measure of DNA synthesis and I believe the data in Fig. 2B is a more accurate reflection of DNA synthesis in these cells.

Limitations of the use of Fluoromicrofluorography as a monitor of S phase.

In this type of analysis, the DNA content of individual cell is measured by fluorescence and conclusions regarding the amount of DNA synthesis in a cell population can be drawn from the number of cells with more than diploid DNA content. This type of analysis is formally analogous to a continuous labeling experiment with  $^3\text{H}$ -thymidine in which the rate of DNA synthesis is estimated from the slope of the incorporation curve. It is more reliable in that it is independent of the limitations of thymidine incorporation such as transport, phosphorylation, and pool dilution.

With the FMF method, a distinction cannot be made between early S, late  $G_1$ , and the  $G_1/S$  boundary. However, such a distinction would only be important after 20 h of purine starvation as the FMF profiles indicate. In general, the experimental data support the interpretation that the cells are arrested in the early part of S. Thus, when purine deprivation is prolonged to 20 h (approximately one generation time) the cells are distributed solely between  $G_1$  and S, with essentially no cells registering as having a  $4n$  complement of DNA. Therefore, some cells must initiate and/or continue DNA synthesis for a time during deprivation but stop before duplicating their entire complement of DNA.

A seeming problem with the FMF profile of starved cells is that at the end of a 20 h starvation period 36% of the cells have been calculated as possessing an S phase DNA content, although 72% of the cells are detected in S by autoradiography. Other workers have noted that early S is a time of low net DNA synthesis and that this period may be mistaken for  $G_1$  if methods other than autoradiography, i.e., total  $^3\text{H}$ -thymidine incorporation or FMF analysis, are used (Klevecz, Keniston and Deaven, 1975). It is possible that purine-deprived V79 pur 1 cells accumulate only a small

amount of additional DNA. In any case, the FMF data obtained in this study emphasize the necessity for evaluating FMF patterns in conjunction with data obtained by more conventional techniques. On the other hand, the use of autoradiography alone does not distinguish between cells that have successfully completed S and those that have not. Thus, failure to use more than one technique for cell cycle analysis may result in misleading conclusions.

It should be noted in Chapter 2, Figure 3, that there is a marked difference in the profiles of the mutant at time zero and at 2 h after purine depletion. The V79 line when grown in optimal conditions has a very short  $G_1$  and  $G_2$  phase. These conditions are obtained when the cells are grown in Ham's F10 medium (time zero). When the cells are grown in less than optimal medium, such as MEM, a  $G_1$  period is observed. The control cells in Figure 3 are maintained in MEM from time zero to the end of the experiment, and the FMF profiles reflect the presence of a measurable  $G_1$ .

As pointed out in Chapter 2, the autoradiography data in Table 1 of that chapter is taken from Figure 2B, and the FMF data was taken from the experiment in Figure 3. The table was constructed to facilitate comparison between the two experiments. The experiments were done at different times, but were both monitored by thymidine incorporation into acid-precipitable material, and the degree of inhibition in the two experiments were practically identical. In fact it should be pointed out that all analyses in this thesis involving the use of depleted mutant cells were monitored by exogenous thymidine incorporation into DNA and if a typical inhibition curve was not obtained under standard conditions of depletion, the experiment was discarded and a new one started.

Finally, the experiment involving measurement of cell DNA content (Fig. 3, p.67) was performed only once. At the time this experiment was done, there was no cytofluorograph available in the Montreal area. The experiment itself, which was essentially the same basic experiment that had been repeated many times in the course of this work (that is, purine depletion was used to inhibit DNA synthesis in the mutant cells), was performed here in Montreal with appropriate controls to insure that the results were similar to those obtained on other occasions. The cells were then treated with propidium iodide under standard conditions and then taken by me to New York where I performed the analysis under the supervision of Dr. Eilen and Dr. Basilico. These two scientists have had extensive experience with the technique. My discussions with them assured me that this analysis was reliable and the results of the experiment were consistent with the other experimental data I had obtained. Considering the inconvenience and expense of the experiment and the absence of any evidence that the result was unreliable, it was decided not to repeat the experiment. Since my controls showed the usual reproducible decrease in thymidine incorporation into the mutant cells under restrictive conditions and the only possible source of error was machine error for which there was no evidence, I am completely confident of the results of this experiment.

#### Analysis of the deoxyribonucleoside triphosphate (dNTP) pools.

When  $G_1$ -arrested cells are stimulated to grow either through serum addition or through virus infection, the dNTP pools are elevated up to 27-fold (Nordenskjold et al., 1970; Adams et al., 1971; Skoog and Nordenskjold, 1971; Weber and Edlin, 1971; Bjursell et al., 1972). In such experiments the investigators worked with synchronized cells. In

the experiments in this thesis, the pool studies were performed on logarithmically growing cells and, because of the vast difference in pool levels in  $G_1$  versus S and  $G_2$  phase cells, it can be argued that our measurements excluded  $G_1$  cells. Furthermore, the distribution of logarithmically growing cells through the cell cycle is such that there is a predominance of cells early in the cycle, compared to the number late in the cycle. In essence therefore, we measured pools mainly in S phase cells. The approach using unsynchronized cells was chosen with this reasoning in mind. In addition, we wished to avoid the pitfalls of measuring pools in cells that might be subjected to unbalanced growth conditions. This is a criticism that can be leveled at any study using synchronized cells and it is particularly pertinent in pool studies, since changes in dNTP pools are very sensitive to experimental manipulation such as might be used to synchronize cells (Hauschka, 1973).

Pool sizes in S phase cells are small; the level of nucleoside triphosphates is sufficient to sustain DNA synthesis for less than 4 minutes (Walters et al., 1973).

Since 1) the control cells were in optimal physiologic conditions for accurate measurement of pool sizes; 2) the kinetics of the cell cycle and the virtual absence of measurable pools in  $G_1$  cells insured that our measurements were essentially in S phase cells; and 3) pool sizes are such that they sustain DNA synthesis for an extremely short time, we feel that the changes seen in the pools in the mutant cells are related to the changes in DNA synthesis. What is to be emphasized is that there is a change in the balance of the pools in the mutant cells under restrictive conditions. This is most clearly demonstrated by the changes in the ratios of the pyrimidine to purine dNTP pools caused by depletion (see Table 1). Thus, although the relationship between the

four dNTPs remains the same, as mentioned in Chapter 2, namely the dCTP pool is the largest and the dGTP pool is the smallest, there are dramatic changes in the ratios of the pyrimidine dNTPs to purine dNTPs. We feel that these changes are related to the inhibition of DNA synthesis observed in the depleted cells not only because the changes in the ratios parallel the inhibition of DNA synthesis seen in *in vivo*, but also because when dNTPs are provided in equimolar amounts *in vitro*, DNA synthesis resumes immediately at near normal rates.

By 20 h post-purine deprivation all the pools have been restored to near control levels. At the same time, DNA synthesis, as measured by total  $^3\text{H}$ -thymidine incorporation, is still at 20-25% of the control. Autoradiography, however, reveals that the proportion of the deprived cells in S is 100% of the control. As it has been explained on numerous occasions above, A/R is the only technique that can detect cells in early S, and presumably the majority of our cells are arrested in early S by 20 h. Therefore, at this point (20 h) the most reliable indication of DNA synthesis is autoradiography and by this criteria DNA synthesis is restored to near normal.

In an attempt to correlate the results obtained from the analyses of the dNTP pools, whole cell autoradiography and  $^3\text{H}$ -thymidine incorporation into acid precipitable material, a summary figure has been plotted (see Figure 1 of this chapter). As it can be seen from this figure, the whole cell autoradiography results correlate best with those for the dNTP pool sizes.

It should be noted that the small decline in the dGTP pool is just as likely as that of the dATP pool to cause DNA synthesis to stop. The  $k_m$ s of both  $\alpha$ - and  $\beta$ -polymerases are quite similar, in the range of 2-13  $\mu\text{M}$  for all four dNTPs, while the  $\gamma$ -polymerase has  $k_m$ s for all four

dNTPs in the range of 0.2-1.0  $\mu$ M (Spadari and Weissbach, 1974). The enzyme that would be least affected by decreasing the purine dNTP pools is the  $\gamma$ -polymerase. However, the enzyme thought to be implicated in DNA replication is the  $\alpha$ -polymerase (Weissbach, 1977).

#### In vitro DNA synthesis.

The method used for the in vitro experiments is that of Reinhard et al., (1977). In that study, optimum requirements for in vitro DNA replication were carefully determined and these requirements were adopted in my studies as well. More specifically, it is clear from the study of Reinhard et al. that the requirements of dNTP concentration are very strict in order for DNA replication to proceed in vitro. (Table IV, p.508, Reinhard et al., 1977, BBA, 474: 500-511.) Thus, if one of the four dNTPs is omitted from the reaction mixture, incorporation of  $^3\text{H}$ -dTTP into acid-insoluble material is dramatically reduced to values of 8 - 16% of the control. If more than one of the dNTPs are omitted the value drops to 3% of the control. Furthermore, if the levels of dNTPs are reduced by 50%, from  $1 \times 10^{-5}$  M to  $5 \times 10^{-6}$  M, the level of vitro synthesis is reduced more than 50%. This experiment was designed to ask one question: What happens to DNA synthesis in the mutant cells if the dNTP levels are not altered by deprivation? The answer is clear - there is little if any inhibition of DNA synthesis. Altering the experimental conditions to mimic the pool level observed in vivo would have resulted in less than optimal DNA synthesis in vitro and would have made the results uninterpretable.

It is worthwhile to speculate as to what would have been the interpretation had we observed a decrease in in vitro synthesis in the mutant cells in the presence of optimum pool sizes. Such a result would have suggested that the inhibition of de novo purine synthesis has a direct

effect on DNA synthesis, such as blocking DNA polymerase or some other enzyme directly concerned with replication. This would have been especially intriguing as it would have opened the possibility that precursor synthesis might regulate DNA replication. This has been suggested in the literature (Housman and Huberman, 1975) but there is no evidence to support it. The results of the present experiment provide evidence against this possibility.

On the other hand, it should be pointed out that no ideal *in vitro* system for mammalian cellular DNA synthesis exists. The particular system used is one of the better ones, but initial synthesis is only 30% of the *in vivo* rate, and less than 3% of the genome is replicated. There is some evidence that initiation of synthesis occurs in this system but it is certainly reduced compared to *in vivo* and the rate of DNA chain growth is also retarded 50% (Hand and Gautschi, 1979). Deficiencies such as these are almost certainly present in all *in vitro* systems, although the one used in the present studies is the only one in which deficiencies in initiation and chain growth have been documented.

These deficiencies are known to investigators in the field, and the results of *in vitro* experiments are interpreted accordingly. In my experiment, a limited question was asked of a limited system. Additional *in vitro* experiments performed under less than optimal conditions would be difficult to interpret. In any case, the best evidence that I am dealing with an S phase population as opposed to a population in late  $G_1$  or at the  $G_1/S$  boundary comes from the *in vitro* studies (chapter 2, Table II and Figure 5). If cells were in late  $G_1$  or at the  $G_1/S$  boundary, the only way to interpret the *in vitro* results would be that near normal initiation is taking place *in vitro*, a situation that does not obtain with this system in hamster cells (Hand and Gautschi, 1979). Thus, in general,  $G_1$  cells do not initiate when triphosphates are added *in vitro*. Supportive evidence



for an S phase population also comes from the pool sizes, which are more suggestive of S phase than  $G_1$  cells.!

### Histone synthesis and DNA synthesis

The relationship of histone synthesis to DNA synthesis and the cell cycle has been presented in the introduction and in chapter 3. Histone, an essential part of chromatin, is thought to be involved in some way with DNA chain elongation in eukaryotes (Weintraub, 1972). It seemed of interest to find out how the overall synthesis of histones was affected by purine deprivation, which apparently caused a drastic inhibition of DNA synthesis. I found that the overall histone synthesis is affected to a lesser extent than DNA synthesis. Although more refined experiments would be necessary in order for conclusions to be drawn, one might speculatively argue that histone synthesis does not seem to be a limiting factor in our system, and that an adequate supply of histones would continue with the DNA newly synthesized by the purine deprived cells and thereby protect it from nuclease attack. Furthermore, as it is also pointed out in chapter 3, a relatively high amount of histone synthesis in comparison to that of DNA would argue in favor of the purine-deprived cells being committed biochemically to the S phase but being unable to successfully duplicate their DNA.

A much better resolution of histones could have been obtained by using an SDS-urea gel as described by Panyim and Chalkley (1969). However, the scope of our experiment was not the detailed analysis of the various histone fractions. We were interested rather to find out in a crude way whether the overall synthesis of histones was affected in the purine-deprived cells and this could be adequately answered by using the method of Winocour and Robbins. With this method the lysine-rich histones H1 (peak 1, MW:21,000), H2A and H2B (peak 2, MW:14,000 and 13,000 respectively)

can be resolved. The latter two, migrate as one peak due to a small difference in the molecular weights.

An alternative method of asking the same question would have been the measurement of radioactive lysine and arginine incorporation into acid-soluble nuclear material (Ensminger and Tamm, 1970). The method used in the thesis provides increased specificity to this analysis. An important point to be raised is that the experimental design was such that the question posed could be answered only by using a double label technique so that the wild-type and mutant cells provided their own internal controls. Double label techniques involving the use of gels require that the gels be sliced. The slicing of gels for analysis as compared to autoradiography (where only single label can be used) results in the marked loss of resolution. Since the important point here was to compare overall histone synthesis before and after purine depletion, the double-label technique was chosen.

A detailed analysis of the synthesis of individual histones was felt to be beyond the scope of this thesis. First, a firm correlation with DNA synthesis has been made only with overall histone synthesis. Post-translational modifications as opposed to synthesis of individual histones occur as cells progress through the cycle. Second, despite the rapid advance in our knowledge of histone chemistry over the past few years, there is still no evidence that they themselves regulate replication (what little evidence there is on their roles in DNA function suggest they may be important in the regulation of transcription).

A related subject that might be worth studying in the future is the relation of chromatin structure and replication in a DNA negative mutant. In normal wild-type cells, a beginning has been made with the studies of Seale's group and Weintraub's group. These investigators have shown that newly synthesised histones are rapidly assembled onto newly replicated

DNA and that histone segregation proceeds conservatively when new DNA is synthesized. A feasible approach in mutant cells in which the complete DNA phenotype is known might be to analyze the fine structure of newly replicated chromatin using high-resolution gels, enzyme digestion studies and electron microscopy.

Experimental drawbacks regarding the characterization of the newly synthesized DNA.

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As another method of analyzing DNA synthesis, I chose to measure the size of DNA made after a short pulse with  $^3\text{H}$ -thymidine and follow the fate of this newly synthesized DNA when it is subsequently incubated in the presence of excess unlabeled thymidine ("pulse-chase" technique). These experiments were analyzed by alkaline sucrose gradients and electrophoresis in agarose gels (Figures 3 and 4, chapter 3). In the sucrose gradient analyses pulse experiments showed clearly that in control and mutant cells, small DNA consistent in size with Okazaki pieces is synthesized. Following the chase in the presence of unlabeled thymidine, the small DNA is apparently not efficiently incorporated into bulk DNA under restrictive conditions in the mutant cells. As pointed out in the discussion of chapter 3, these results are consistent with the interpretation that there is a defect in elongation at the level of either ligation or gap-filling.

There are drawbacks to these experiments. First, calculation of the total  $^3\text{H}$ -counts in bulk DNA in the control pulse-chase experiment (panel c, Figure 3) indicates that the increase in bulk DNA following the chase is only 70% as compared to the 700% to 800% that was achieved in the purine-deprived (panel b, Figure 3) and in the control in Figure 1 of the Appendix. Thus the experiment is flawed and must be interpreted with caution. Nevertheless, since there is an increase in bulk DNA in the control (panel d, Figure 3), I am forced to draw the conclusion that mutant Okazaki pieces

are less efficiently incorporated into bulk. Other effects may also be operative and cannot be completely excluded. Thus I cannot completely rule out the possibility that mutant and wild-type Okazaki pieces are degraded. Therefore, the defect in elongation may be a minor one, but it is still definitely present. This is only one of several defects I have demonstrated in DNA replication in the mutant cells. The work with fiber autoradiography shows that DNA initiation and replication fork movement are also inhibited. Multiple defects in the replication process fit nicely with the alterations demonstrated in the studies on the dNTP pools in Chapter 2, and point clearly to the importance of the precursor pools in all aspects of DNA replication.

The gel electrophoresis experiments in Figure 4 were attempted to expand this sort of analysis. They were not completely successful. It is important to point out that I attempted here to substitute electrophoresis for alkaline sucrose gradient analysis and labeled cell extracts were not purified, but rather lysed immediately before electrophoresis. As a result, DNA of high molecular weight was unable to enter the gels completely, and good pulse chase kinetics were not demonstrated. The conclusions that can be drawn from these experiments are only that the DNA of Okazaki fragment size persist in the control cells. Again this could be due to defective elongation in the mutant, or breakdown of the DNA in the control. However, taken together with the results of the standard sucrose gradient analyses and the fiber autoradiography experiments, the most likely interpretation is defective elongation in the mutant cells.

The heterogeneity in Okazaki fragment size evident in the gels is not unprecedented. Perlman and Huberman (1977) and Anderson et al. (1977) have also observed heterogeneity of this sort when purified Okazaki fragments were analyzed by agarose gel electrophoresis.

Had these experiments been more successful, that is, had the bulk DNA entered the gel completely, the analysis by electrophoresis would

have provided an excellent alternative to standard alkaline sucrose gradient analyses. There would have been the tremendous advantage of reproducibility, since multiple samples could have been run in the same gel. To this would have also been added the advantage of speed of analysis, especially if the technique of fluorography could have been used. Future experiments along this line may be worthwhile. They should probably be tried with extremely diluted gels (0.2%) run on a horizontal electrophoresis apparatus. Of course, controls involving the analysis of purified Okazaki fragments and DNA molecules of defined size will also have to be run.

Finally, it should be pointed out that the molecular weight marker used was the relaxed circular double stranded form of  $\phi$ X174 (RFII), which under alkaline conditions sediments at 16s and whose calculated molecular weight is  $1.7 \times 10^6$  (Studier, 1965). Based on these values we estimated the material on the top of our gradient to be 4-5 S. Given the fact that only one molecular weight marker was used this value is only a rough estimate.

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TABLE 1 - Ratios of pyrimidine to purine dNTP pools in purine-deprived and purine-supplemented (control) V79 pur 1 cells. The dNTP pools were measured according to the method described in the experimental procedures of Chapter 2, and the actual pool sizes used to calculate these ratios are presented in Figure 4 of Chapter 2.

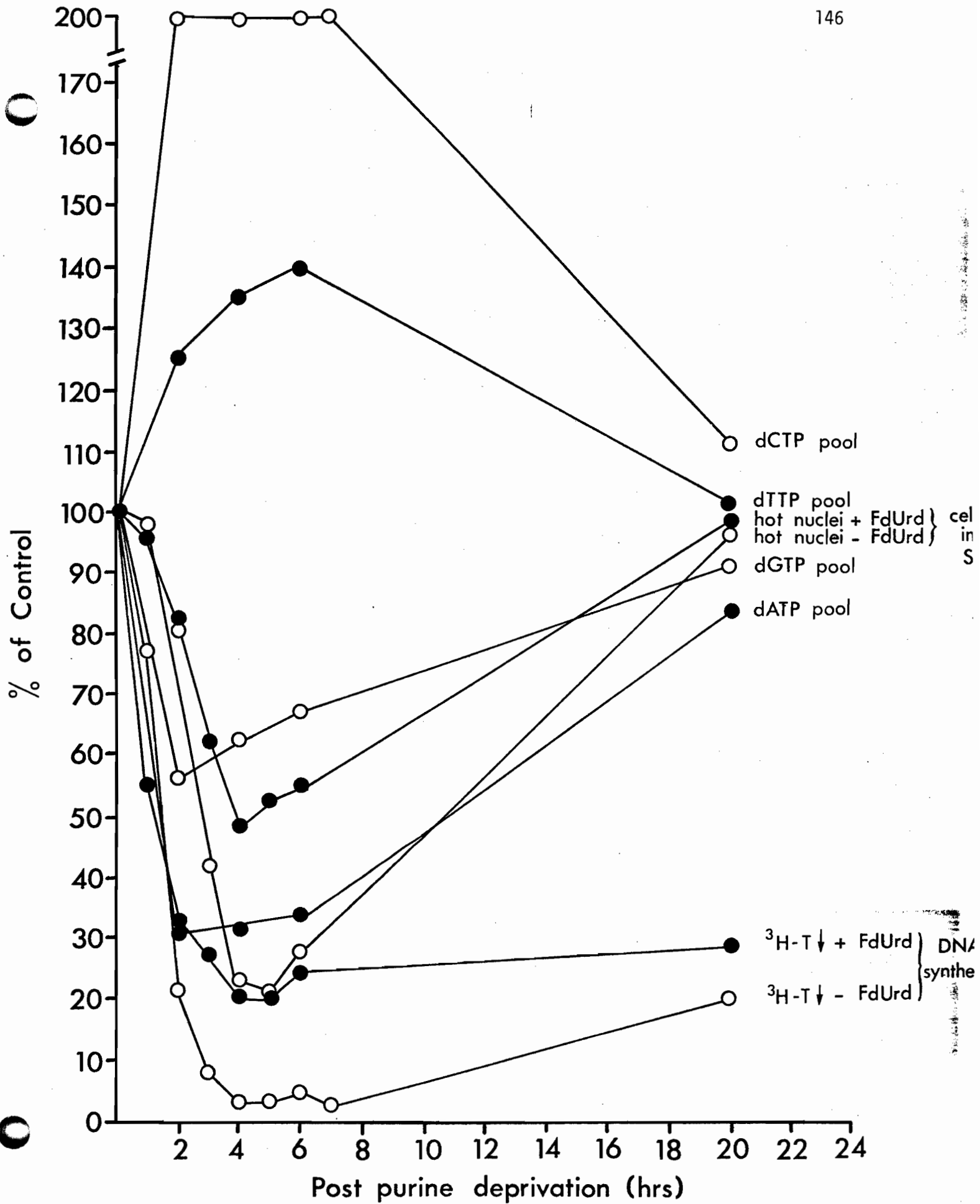
Table 1 - Ratios of pyrimidine to purine dNTP pools in purine-depleted (D) and control (C) V79 pur 1 cells.

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Time (h)	<u>dCTP/dATP</u>		<u>dCTP/dGTP</u>	
	<u>D</u>	<u>C</u>	<u>D</u>	<u>C</u>
2	14	3	94	30
4	15	3	82	24
6	14	3	82	31
	<u>dTTP/dATP</u>		<u>dTTP/dGTP</u>	
2	3	1	21	12
4	4	1	23	12
6	4	1	22	10

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FIGURE 1 - Summary diagram showing the results of the measurements of  $^3\text{H}$ -thymidine incorporation into acid insoluble material and into S phase nuclei (see also Figure 2, chapter 2), as well as the results of the dNTP pool analyses (see also Figure 4, chapter 2) in the purine-deprived V79 pur 1 cells. All results are expressed as percent of control.



## OVERALL CONCLUSION AND PERSPECTIVES

Our study of the purine auxotrophic mutant V79 pur 1 revealed that in the absence of de novo purine biosynthesis semiconservative DNA synthesis is markedly inhibited and the cells are unable to traverse the S phase and synthesise a full DNA complement. The rate of replication fork movement as well as the elongation of nascent DNA chains are inhibited but the limited DNA synthesis which does proceed in the purine deprived cells suggests that chromosomal DNA synthesis can be initiated. The size of the purine deoxyribonucleoside triphosphate pools decreases while that of the pyrimidine pools increases. The inhibition of DNA synthesis caused by the block to purine synthesis may be reversed by reestablishing the balance in the nucleotide pools.

There are a number of possible explanations for the observed defect of the V79 pur 1 cells that involve mainly the supply of precursor molecules. Thus, DNA chain elongation may be dependent on a specific ligase that requires the presence of all four dNTPs in balanced amounts for maximum ligating efficiency. Alternatively, the defective DNA synthesis could be due to the reduced rate of DNA chain growth which might cause the production of DNA segments that cannot be recognized by normal enzymes.

Although many advances have been made toward understanding the process of DNA replication, many steps still remain unclear. The enzymology of the eukaryotic replication system needs to be worked out in detail and such a task would be greatly facilitated through the isolation and study of mutants defective in some aspect of DNA synthesis. Thus, although many enzymes of DNA metabolism are now characterized, mutant isolation and genetic analysis will be necessary in order to assign specific roles to the many components required for replication. Few such eukaryotic mutants have been isolated up to now. The majority of these that exhibit defective DNA

synthesis are mutant in a non-S-phase function. Of particular interest, however, are the S-phase mutants that are deficient in DNA replication per se. In the study presented here we have described such a mutant and we have presented an analysis of its particular defect in DNA synthesis. We hope that this mutant in conjunction with other S-phase mutants that are already available and others still that will become available in the future, will be used as genetic tools in the elucidation and understanding of DNA synthesis.

APPENDIX



TABLE 1:

Cells were seeded into petri dishes in F10 plus 5% FCS.

After growth overnight half the dishes received medium without purines and the other half received medium supplemented with 30  $\mu$ M hypoxanthine (control). At the time points indicated samples were pulsed with  $^3$ H-thymidine (1  $\mu$ Ci/ml) for 15 min.

The data are expressed as percent incorporation of the corresponding control cells maintained in hypoxanthine-supplemented medium.

Table 1 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material in V79 pur 1 cells Depleted of purines for 5, 6 or 7 h and then supplemented with Hypoxanthine ( $30\ \mu\text{M}$ ; repleted) for 1 h.\*

Time (h)	$^3\text{H}$ -thymidine Incorporation (% of control)	
	Depleted	Repleted
5	6	96
6	5	95
7	5	95

\* Data appearing in this table supplement those of Figure 2 in Chapter 2.

TABLE 2:

The cells were prepared as in Table 1 of this appendix and were identically treated at the time points indicated.

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Table 2 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material  
in Purine-Depleted V79 pur 1 Cells.\*

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Time (h)	$^3\text{H}$ -thymidine Incorporation (% of control)
24	16
34	12
48	6
72	.5

---

\* Data appearing in this table supplement those of Figure 2 in  
Chapter 2.

TABLE 3:

The cells were prepared and treated as in Table 1 of this appendix.

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Table 3 -  $^3\text{H}$ -thymidine Incorporation into Acid Insoluble Material in Purine-Depleted V79 (wild-type) Cells.\*

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Time (h)	$^3\text{H}$ -thymidine Incorporation (% of control)
2	100
4	104
6	109

---

\*Data appearing in this table supplement those of Figure 2 in Chapter 2.

TABLE 4:

Effect of purine deprivation on dNTP pool size in V79 (wild-type) cells. Following overnight growth in complete (F10) medium the cultures were shifted to purine-deprived or purine-supplemented medium and the dTTP, dCTP, dGTP and dATP pools were determined at the time points indicated.

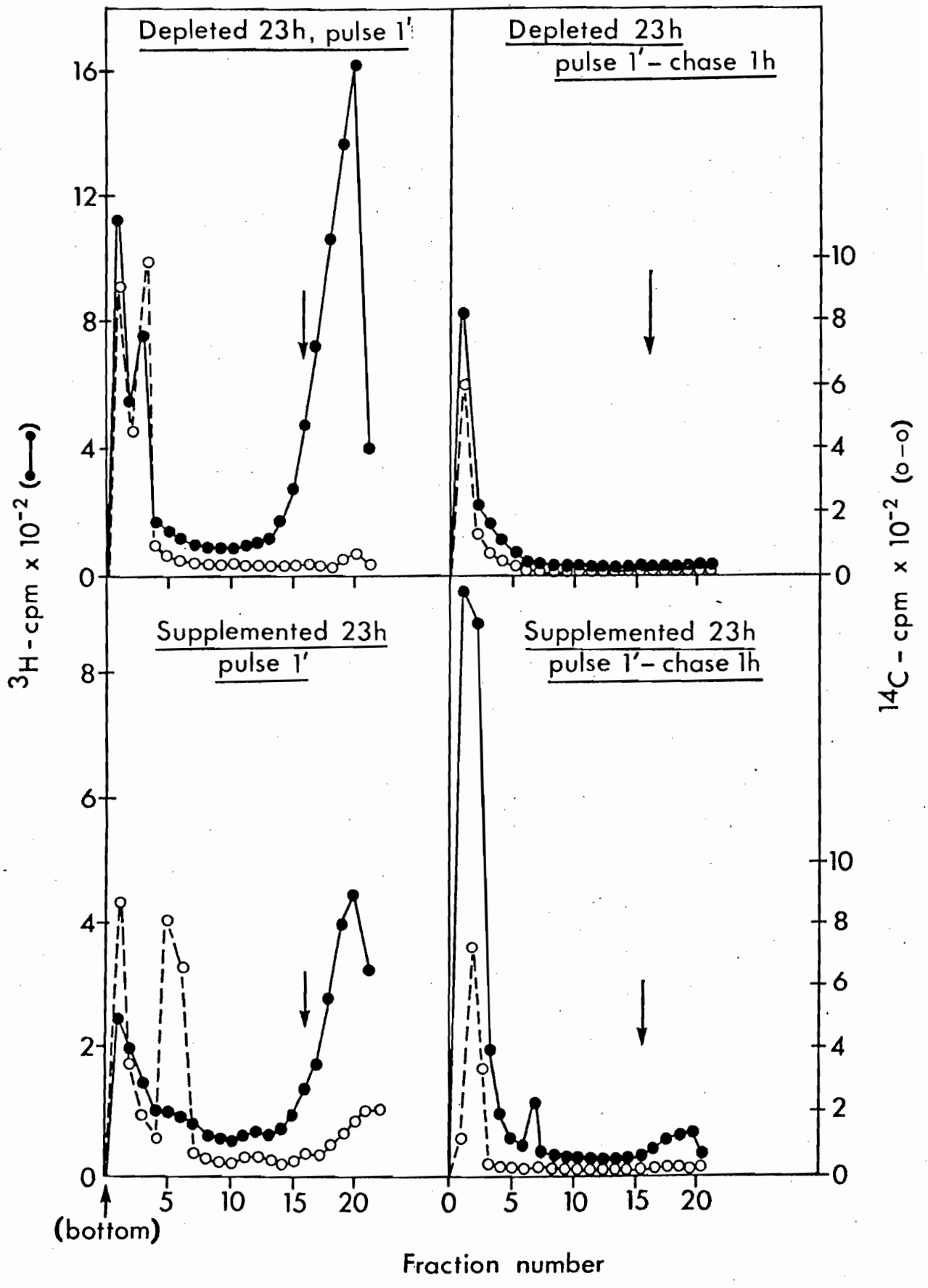
Table 4 - Deoxyribonucleoside Triphosphate (dNTP) Pool Size  
in V79 (wild-type) Cells.\*

Time (h)	Medium	dNTP (picomoles/10 <sup>6</sup> cells)			
		dTTP	dCTP	dGTP	dATP
0	-	70	130	6	20
6	Depleted	63	170	5	41
	Control	71	160	6	43

\* Data appearing in this table should be compared to those  
of Figure 4 in Chapter 2.



Figure 1. Alkaline sucrose gradient centrifugation of DNA synthesized by purine-deprived V79 pur 1 cells in pulse-chase studies. Cells were prelabeled with  $^{14}\text{C}$ -thymidine (0.01  $\mu\text{Ci/ml}$ ; 50 Ci/mmol) in F10 plus 5% FCS. After growth overnight, half the cultures received medium without purines and the other half received medium supplemented with 30  $\mu\text{M}$  hypoxanthine (control). After 23 h cells were pulse-labeled with  $^3\text{H}$ -thymidine ( $2 \times 10^{-6}$  M; 50 Ci/mmol) for 1 min and were processed either immediately or after 1 h chase with unlabeled thymidine ( $2 \times 10^{-5}$  M). Arrow indicates position of  $\phi\text{X174}$  marker DNA run in a parallel gradient. ( • ),  $^3\text{H}$ -cpm; ( o ),  $^{14}\text{C}$ -cpm.



CLAIM TO ORIGINAL RESEARCH

In the study presented in this thesis the following observations were made for the first time:

When the pathway leading to the biosynthesis of purines de novo is impaired the purine-depleted cells:

- 1) show an increase in size of the pyrimidine deoxyribonucleoside triphosphate (dNTP) pools and a decrease in size of the purine dNTP pools.
- 2) are arrested in S phase
- 3) are capable of DNA synthesis in vitro at near normal rates immediately after exposure to an exogenous supply of nucleotides
- 4) are capable of semiconservative DNA synthesis in vivo
- 5) have a reduced rate of DNA replication fork movement

