

THE UNIVERSITY OF MANITOBA

A STUDY OF THE METABOLISM OF A NOVEL DINUCLEOSIDE
POLYPHOSPHATE (HS3) FOUND IN MAMMALIAN CELLS:
POSSIBLE REGULATION OF NUCLEIC ACID BIOSYNTHESIS
BY HS3 DURING STEP-DOWN GROWTH CONDITIONS

BY

SWEE HAN GOH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULLFILMENT OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

APRIL, 1979

A STUDY OF THE METABOLISM OF A NOVEL
DINUCLEOSIDE POLYPHOSPHATE (HS3)
FOUND IN MAMMALIAN CELLS : POSSIBLE
REGULATION OF NUCLEIC ACID BIOSYNTHESIS
BY HS3 DURING STEP-DOWN GROWTH
CONDITIONS.

BY

SWEE HAN GOH

A dissertation submitted to the Faculty of Graduate Studies of
the University of Manitoba in partial fulfillment of the requirements
of the degree of

DOCTOR OF PHILOSOPHY

© 1979

Permission has been granted to the LIBRARY OF THE UNIVER-
SITY OF MANITOBA to lend or sell copies of this dissertation, to
the NATIONAL LIBRARY OF CANADA to microfilm this
dissertation and to lend or sell copies of the film, and UNIVERSITY
MICROFILMS to publish an abstract of this dissertation.

The author reserves other publication rights, and neither the
dissertation nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.

To Siew See and my parents

Acknowledgements

I would like to thank Dr. Herb B. LeJohn for his advice, interest and patience during the course of this research programme. The friendship and constructive criticisms of Linda, Renate, Dave, Glen, Rob and other members of the Dept. of Microbiology are gratefully acknowledged.

I would also wish to express my deepest gratitude to Dr. Jim Wright for his generosity and invaluable advice with mammalian tissue culture techniques and the gift of numerous cell lines used for this study.

The counsel of Drs. I. Suzuki and Peter Loewen is much appreciated.

ABSTRACT

A novel dinucleoside polyphosphate (HS3) previously found in various fungi has now been detected in numerous cultured mammalian cell lines. Physical and chemical analyses of HS3 (McNaughton et. al. 1978) show that the molecule consists of a glutamyl-ADP-sugar moiety which is covalently linked to a UDP-mannitol-tetraphosphate.

Physiological studies demonstrated that as long as de novo purine biosynthesis was blocked (by nutrient deprivation, drugs or mutation) and no exogenous purines (and some pyrimidines) were supplied, HS3 accumulation took place. For example, the addition of de novo purine biosynthesis inhibitors [azaserine (50 µg/ml) or MTX (1 µM)] resulted in HS3 synthesis as did the withdrawal of glutamine (2 mM). The addition of hypoxanthine, inosine, adenine, adenosine, guanosine, uridine and cytidine, but not thymidine, (all at 0.3 mM) strongly suppressed HS3 accumulation in glutamine-starved CHO cells. CHO purine salvage mutants (HGPRT⁻) failed to do likewise when supplied with high concentrations of hypoxanthine, guanosine and inosine (0.3-2.0 mM). A CHO mutant (GAT⁻-auxotrophic for adenosine, thymidine and glycine) with blocked de novo purine biosynthesis, accumulated HS3 only when adenosine (0.1 mM), but not glutamine, was withdrawn from its growth medium. Thus

HS3 accumulation may be related to a lack of either precursors for purine nucleotide biosynthesis or of purine nucleotides themselves.

The rate of HS3 synthesis in CHO WT cells increased 5-6 fold shortly after glutamine withdrawal and was maintained at that level for at least 6 hr before declining slowly back to the control rate by 22 hr. The pool sizes of HS3 increased and decreased in concert with rate changes. The replenishment of glutamine and adenosine to starved CHO WT and CHO GAT⁻ cells respectively, resulted in an immediate depletion of the accumulated HS3.

An inverse relationship was observed between nucleic acid (DNA and RNA) synthesis and HS3 accumulation. When DNA and RNA synthesis decreased, HS3 synthesis increased and vice versa. No such correlation was apparent for protein synthesis.

The inhibition of protein synthesis by either puromycin (100 µg/ml) or cycloheximide (10 µg/ml) or that of RNA synthesis by actinomycin D (1 µg/ml), stimulated HS3 depletion in glutamine-starved CHO WT cells.

Both fungal and mammalian HS3 were found to be equally potent inhibitors of in vitro and in vivo

mammalian RNA synthesis. Also, studies by Lewis et. al. (1977) showed that HS3 strongly inhibited partially purified ribonucleotide reductase from CHO cells. Thus the decrease in DNA synthesis during periods of rapid HS3 accumulation may be the indirect result of a deficiency in deoxyribonucleotides. The data suggest that HS3 may be involved in the regulation of nucleic acid biosynthesis in mammalian cells.

TABLE OF CONTENTS

	PAGE
Acknowledgements.....	iii
Abstract.....	v
Table of Contents.....	viii
List of Tables.....	xvii
List of Figures.....	xix
Abbreviations.....	xxii
Introduction.....	xxvi
Historical	
Tissue Culture.....	2
Development of Tissue Culture	
Techniques.....	2
Growth Requirements and	
Characteristics of Cultured	
Mammalian Cells.....	5
Mammalian Cells: RNA Biosynthesis and	
Amino Acid Withdrawal.....	9
Physiology, Biochemistry and Possible Roles	
of Polyphosphates and Unique Phosphory-	
lated Nucleosides in Procaryotic and	
Eucaryotic Cellular Regulation.....	11

	PAGE
Polyphosphates.....	12
Adenosine 3':5' Cyclic Monophosphate..	16
Biosynthesis, Catabolism and Possi- ble Regulatory Role(s).....	16
Cyclic AMP in Bacteria.....	18
Cyclic AMP in the Fungi.....	20
Cyclic AMP in Mammalian Cells in culture.....	22
Guanosine 3':5' Cyclic Monophosphate..	24
Bacterial MS (ppGpp and pppGpp)	
Nucleotides.....	26
The Stringent Response.....	26
<u>In Vivo</u> Synthesis of ppGpp and pppGpp.....	27
<u>In Vitro</u> Synthesis of ppGpp and pppGpp.....	32
Effects of ppGpp and pppGpp on Cell- ular Enzymes, Translation and Transcription <u>In Vitro</u>	34
Influence of Carbon and Nitrogen Sources on ppGpp, pppGpp and rRNA Metabolism.....	37
HPN Compounds.....	39
Diguanosine Polyphosphates.....	41

	PAGE
Diadenosine Polyphosphates.....	42
Triadenosine Pentaphosphate.....	43
Dinucleoside Polyphosphates (HS).....	44
Other Polyphosphorylated Nucleosides.	48
Mammalian Purine and Pyrimidine Metabolism.	49
Regulation of <u>De Novo</u> Purine Biosynth- etic Enzymes in Mammalian Cells and Tissues.....	49
PRPP Synthetase and Intracellular PRPP Levels.....	52
5-Phosphoribosyl-1-Pyrophosphate Amidotransferase.....	54
Regulation of AMP and GMP Synthesis from IMP.....	57
Induction, Repression and Derepression of <u>De Novo</u> Purine Biosynthetic Pathway Enzymes.....	58
Regulation of <u>De Novo</u> Pyrimidine Bio- synthesis in Mammalian Cells and Tissues.....	59
Human Genetic Disease and Defective Purine and Pyrimidine Metabolism.....	63

	PAGE
Gout.....	63
Lesch-Nyhan Syndrome.....	64
Immunodeficiency Syndromes.....	65
Oroticaciduria and Xanthinuria....	69
 Materials and Methods	
Organisms.....	72
Mammalian Cell Lines.....	72
Fungal Cells.....	72
Growth Media, Sera and Culture-wares.	73
Radioisotopes.....	73
Miscellaneous Materials.....	73
Methods.....	75
Growth of Cell Lines.....	75
Growth, ³² P-Orthophosphate Labelling and Formic Acid Extraction of Cells for Analysis of HS Polyphosphorylated Nucleotides During Various Growth Conditions.....	76
Chromatography of ³² P-Labelled Formic Acid Extract from Mammalian cells....	77
DNA, RNA and Protein Analysis.....	78
DNA Pulse Labelling Experiments...	79

Protein Pulse Labelling	
Experiments.....	79
Continuous RNA Labelling	
Experiments.....	80
Determination of ¹⁴ C-Adenine Incorporation into Nucleosides and Nucleotides.....	81
Determination of ATP Levels Using the Firefly Luciferin/Luciferase Method..	81
Isolation of HS3 From <u>Achlya</u> and CHO WT cells.....	83
Isolation of DNA-Dependent RNA Polymerases From CHO WT Cells.....	84
Cell Permeabilization and Assay for RNA Synthesis.....	87
Assay of RNA Synthesis By Permeabilized CHO WT Cells In the Presence of <u>Achlya</u> HS3.....	88
Results	
HS3 Synthesis by CHO WT Cells.....	90
Effect of Varying Concentrations of L-Glutamine on HS3 and GTP Synthesis by CHO WT Cells.....	99

	PAGE
Effect of L-Glutamine or L-Isoleucine Deprivation on HS3 Metabolism by CHO WT Cells.....	102
Effect of L-Glutamine, Adenosine and Thymidine Deprivation on HS3 Metabolism by CHO GAT ⁻ Cells.....	102
Effect of L-Glutamine Withdrawal and the Replenishment with Purines on HS3 Metabolism by CHO WT and CHO Purine Salvage Mutant Cells.....	105
Influence of Purine and Pyrimidine Compounds, Methotrexate and Azaserine on the Accumulation and Depletion of HS3 in Three CHO Cell Lines Incubated in the Presence or Absence of L-Glutamine.....	109
Determination of ATP Levels in CHO WT Cells After Exposure to Various Growth Conditions and Metabolic Inhibitors.....	110
Effects of Glutamine Deprivation or Sufficiency on ¹⁴ C-Adenine Incorporation into Adenine Nucleoside	

	PAGE
and Nucleotides by CHO YHD 13 Cells..	113
Effects of L-Glutamine and Adenosine Deprivation and Replenishment on Rate of Accumulation and Pool Size of HS3..	115
Fate of ³² P-Labelled HS3 Accumulated by CHO WT Cells During Glutamine Deprivation Following ³² Pi with- drawal From the Incubation Medium.....	125
Time Course Studies of the Effects of 5-Fluorouracil on Elevated HS3 Levels Accumulated During L-Glutamine Starvation or Methotrexate Treatment..	128
Effect of L-Glutamine and Adenosine Starvation on Protein Synthesis.....	132
Effect of L-Glutamine and Adenosine Deprivation on Rate of ³ H-Thymidine Incorporation Into DNA.....	135
Effect of L-Glutamine or Adenosine With- drawal and Replenishment on RNA Accumulation by CHO WT and CHO GAT ⁻ Cells.....	139
Effects of L-Glutamine and Adenosine Deprivation and Replenishment on the Growth of CHO WT and CHO GAT ⁻ Cells...	140

	PAGE
Effects of <u>Achlya</u> and CHO WT HS3 on Partially Purified CHO WT Cell DNA- Dependent RNA Polymerases.....	146
Effect of Purified <u>Achlya</u> HS3 on the Incorporation of ³ H-UTP into RNA by Permeabilized CHO WT Cells.....	149
Effects of Antibiotics on HS3 Synthesis..	155
Survey of HS3 Metabolism by Various Cultured Mammalian Cell Lines.....	159
 Discussion	
HS3 Synthesis by CHO WT Cells.....	166
HS3 Accumulation: The Possible Involvement of Other Nutritional Changes Besides Glutamine Deprivation.....	167
Effect of Glutamine Deprivation and Metabolic Inhibitors on GTP and ATP Levels.....	174
HS3 Metabolism: Effect of Antibiotics....	176
HS3 Levels: Elevation by MTX Treatment or Glutamine Deprivation and Depletion by 5-FU.....	178
HS3 as a possible Cellular Regulator: Changes in Rate of Accumulation and Pool Size of HS3.....	182

	PAGE
Intracellular HS3 Levels: Correlation with Protein, DNA and RNA Synthesis and Growth.....	184
Protein Synthesis.....	184
DNA Synthesis.....	185
RNA Synthesis.....	186
Growth.....	190
Possible Effects of HS3 and Nucleoside Triphosphate Levels on Nucleic Acid Synthesis.....	191
Glutamine Availability and HS3 Metabolism by Various Cultured Mammalian Cell Lines.....	194
Conclusion.....	198
Bibliography.....	199

LIST OF TABLES

TABLE	PAGE
1. R_f values of nucleotides	97
2. Comparison of chemical and physical properties of <u>Achlya</u> and mammalian HS3	98
3. Effect of L-glutamine, adenosine and thymidine availability on HS3 metabolism by CHO GAT ⁻ cells	106
4. Effect of L-glutamine and exogenous purines on intracellular HS3 levels in CHO WT and CHO purine salvage mutants	108
5. Influence of purine and pyrimidine compounds, methotrexate and azaserine on HS3 metabolism in CHO WT and purine salvage mutant (YH 21 and YHD 13) cells incubated in growth medium with or without supplemented L-glutamine.....	111-112
6. Determination of ATP concentrations in CHO WT cells after exposure to different growth conditions and metabolic inhibitors.....	114
7. (a) Effect of L-glutamine withdrawal and replenishment on rates of ³ H-TdR incorporation into DNA in CHO WT cells....	137

TABLE	PAGE
(b) Effect of adenosine withdrawal and replenishment on rates of ^3H -TdR incorporation into DNA in CHO GAT ⁻ cells.....	138
8. Effect of <u>Achlya</u> and CHO WT cell HS3 on the <u>in vitro</u> activities of DNA-dependent RNA polymerases from CHO WT cells.....	150
9. HS3 inhibition of RNA synthesis in permeabilized CHO WT cells.....	154
10. Effect of cycloheximide and actinomycin D on HS3 levels on CHO WT cells.....	158
11. Determination of levels of ^{32}P i labelled HS3 in various mammalian cell lines cultured with or without 2 mM L-glutamine.....	162

LIST OF FIGURES

FIGURE	PAGE
1. Schematic diagram of the <u>de novo</u> purine biosynthetic pathway.....	50
2. Schematic diagram of the <u>de novo</u> pyrimidine biosynthetic pathway.....	51
3. One-dimensional autoradiogram of ^{32}P labelled formic acid extracts from CHO WT cells incubated in the presence or absence of glutamine.....	93
4. Two-dimensional autoradiogram of ^{32}P labelled formic acid extract from glutamine-starved CHO WT cells.....	95
5a&b. Glutamine concentration-dependent effect on ^{32}P -labelled HS3 and GTP levels in CHO WT cells.....	101
6. L-Glutamine and L-isoleucine starvation effect on HS3 accumulation by CHO WT cells.....	104
7. Incorporation of ^{14}C -adenine into adenosine and adenosine nucleotides by L-glutamine-deprived or supplemented YHD 13 CHO cells.....	118

FIGURE	PAGE
8. L-Glutamine availability and rates of HS3 synthesis by CHO WT cells.....	120
9a&b. Effect of L-glutamine or adenosine deprivation on intracellular HS3 pool sizes in CHO cell lines.....	122
10a&b. Effect of nutrient (L-glutamine or adenosine) replenishment on accumulated HS3 in CHO cell lines.....	124
11. Fate of ³² Pi-labelled HS3, accumulated by CHO WT cells during L-glutamine deprivation, following ³² Pi withdrawal from the incubation medium.....	127
12a, b&c. Effect of 5-FU on HS3 accumulated by CHO WT cells and L5178Y mouse leukemia lymphoblasts during L-glutamine deprivation or methotrexate treatment.....	131
13a&b. Effect of L-glutamine or adenosine deprivation on protein synthesis by CHO cell lines.....	134
14a&b. Effect of L-glutamine or adenosine availability on RNA synthesis by CHO cell lines.....	142

FIGURE	PAGE
15a&b. Effect of L-glutamine or adenosine availability on the growth of CHO cell lines.....	145
16. DEAE-Sephadex A-25 chromatographic profiles of DNA-dependent RNA polymerases isolated from CHO WT cells....	148
17. Time-course study of the incorporation of ³ H-UTP into RNA by permeabilized CHO WT cells.....	153
18. Effect of antibiotics on HS3 accumulated during L-glutamine deprivation of CHO WT cells.....	157
19a&b. Two-dimensional autoradiograms of formic acid extracts from ³² Pi labelled human foreskin fibroblasts incubated with and without L-glutamine.....	164

ABBREVIATIONS

ADA	adenosine deaminase
ADP	adenosine 5'-diphosphate
α -MEM	alpha-minimal essential medium
AMP	adenosine 5'-monophosphate
APRT ⁻	adenine phosphoribosyltransferase deficient
ATP	adenosine 5'-triphosphate
cAMP	cyclic 3',5'-adenosine monophosphate
cGMP	cyclic 3',5'-guanosine monophosphate
CHO	Chinese hamster ovary
cm	centimetre
cpm (CPM)	counts per minute
dATP	deoxyadenosine 5'-triphosphate
dCDP	deoxycytidine 5'-diphosphate
DFCS	dialysed fetal calf serum
dGTP	deoxyguanosine 5'-triphosphate
FCS	fetal calf serum
5-FdUMP	5-fluorodeoxyuridine 5'-monophosphate
fig.	figure
5-FU	5-fluorouracil
GDP	guanosine 5'-diphosphate
gln	glutamine

GMP	guanosine 5'-monophosphate
GTP	guanosine 5'-triphosphate
HCl	hydrochloric acid
HGPRT ⁻	hypoxanthine/guanine phosphoribosyltransferase deficient
hr	hour
IMP	inosine 5'-monophosphate
M	molar
min	minute(s)
ml	millilitre
mM	millimolar
m mol	millimole
MTX	methotrexate
MW	molecular weight
OMP	orotidine 5'-monophosphate
PEI	polyethyleneimine
Pi	inorganic phosphate
ppGpp	guanosine 3'-diphosphate, 5'-diphosphate (MS I)
pppGpp	guanosine 3'-triphosphate, 5'-diphosphate (MS II)
PRA	phosphoribosylamine
PRPP	5-phosphoribosyl-1-pyrophosphate
TCA	trichloroacetic acid
TdR	thymidine

TEAB	triethylammonium bicarbonate
Tris	tris (hydroxymethyl) amino methane
uCi	microcurie
UDP	uridine 5'-diphosphate
ug	microgram
UMP	uridine 5'-monophosphate
UTP	uridine 5'-triphosphate
UV	ultraviolet
WT	'wild type'
(+)	supplemented
(-)	<u>not</u> supplemented

INTRODUCTION

It is apparent now that complex biochemical regulatory functions, at the enzyme level, have been evolved in living organisms. Yet it is evidently desirable that the diverse cellular activities should be coordinated in some fashion so as to ensure balanced growth during periods of rapid nutrient changes in the organism's growth environment. Since the isolation and characterization of various small yet unique nucleotides from both procaryotes and eucaryotes, there has been an increasing interest in attempting to understand the functions of these molecules in the regulation of growth.

The data on the role(s) of cyclic adenosine monophosphate and bacterial guanosine nucleotides (ppGpp and pppGpp) in procaryotic growth regulation is somewhat convincing. The involvement of ppGpp and pppGpp in regulating transcription during the bacterial 'stringent response' is clear. To date, however, no definitive data have been presented to demonstrate the existence of ppGpp and pppGpp in cultured mammalian cells. Cyclic adenosine monophosphate and cyclic guanosine monophosphate have been implicated in regulating eucaryotic growth. Other