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

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

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To Siew See and my parents

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ABSTRACT

A novel dinucleoside polyphosphate (HS3) prevously 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glycine) with blocked de novo purine biosynthesis, accumulated HS3 only when adenosine (0.1 mM), but not glutamine, was withdrawn from its growth medium.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 accumualtion 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.

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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
(-)	not 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

nucleotide-containing compounds have also been discovered and purported to have important cellular functions. However, diguanosine tetraphosphate has been found to be limited to some crustaceans while the function of diadenosine tetraphosphate, which seemingly is more universal in occurrence, has yet to be demonstrated. It is apparent that not all cellular responses are mediated by these aforementioned compounds. Among these responses is the coordinated physiological changes associated with the removal of an essential amino acid from the growth medium of cultured mammalian cells, a process commonly known as the 'pleiotypic response'.

In 1975, three rather unique dinucleoside polyphosphates were reported to exist in various fungi. These compounds were given the pseudonyms HS1, HS2 and HS3. One of them, HS3, was partially characterized recently as consisting of a glutamyl-ADP-sugar moiety covalently linked in an unknown manner to a UDP-mannitol-tetraphosphate. In vivo and in vitro studies have demonstrated that there is an apparent correlation between changes in the concentrations of these dinucleoside polyphosphates and the rates of RNA and DNA synthesis. Their possible

role(s) in fungal sporulation has also been implicated. It is thus likely that these hitherto unknown compounds may be involved with cellular regulation during adverse growth conditions.

A study of one of these dinucleoside polyphosphates, HS3, found in cultured mammalian cells constitutes the basis of this thesis. The aims of this research undertaking are essentially threefold; firstly, to establish the presence of one of these HS molecules (HS3) in various cultured mammalian cell lines. Secondly, to understand the physiology involved in the metabolism of HS3 in various 'wild-type' and mutant cell lines and thirdly to observe the relationship between changes in the levels of intracellular HS3 and activities of various cellular macromolecular processes during different growth conditions. In the course of this study, it became apparent that HS3 metabolism is indivisibly related to purine-pyrimidine and nucleic acid metabolism. It is hoped that this thesis will, in some very small way, contribute indirectly to a better understanding of purine-pyrimidine metabolism and lead to a better appreciation of some of the human genetic diseases that are related to purine and pyrimidine dysfunction.

HISTORICAL

(1) Tissue Culture

(a) Development of Tissue Culture Techniques

The development of mammalian cell culture techniques has provided scientists with a powerful tool to probe the intricacies of the structure and metabolic processes of clonal mammalian cells. The basic tools of modern day cell culturists consists of glass and plastic substrata, defined growth media and various types of sera. The early problems encountered in attempts to establish permanent cell lines in vitro were two-fold. Firstly, a proper growth-supporting environment had to be formulated and secondly the difficulty in obtaining cell clones from a single cell for genetic studies had to be overcome.

In 1912, Carrel (1912) reported his success in keeping a chicken muscle explant viable and proliferating for many years using a growth medium composed of Ringer's salt solution (Ringer 1882), chicken plasma and muscle extract. Until the early 1950s, this medium and others of similar composition were the primary types of growth media used for cell culture. Despite the use of such complex media, large numbers of single clones could not

be generated until Sanford, Earle and Likely (1948) showed that the growth medium had to be first 'conditioned' before cells seeded at a very low density will proliferate. This was achieved by successfully culturing single cells in very small volumes of growth medium. Alternatively, Puck and Marcus (1955) reported that through the use of an irradiated layer of 'feeder' cells, most of the cells from a HeLa cell (Gey et. al. 1952) population could be cloned. These results suggested that the cloning procedure could be more successful and simplified if the various nutrients essential for growth could be identified. This led to the rapid development of chemically defined growth media. Eagle (1955a, 1955b) was the first to successfully formulate a defined mixture of nutrients for the propagation of mammalian cells in culture. The medium consisted of a mixture of thirteen essential amino acids, vitamins, cofactors, salts, carbohydrates and small amounts of either human or dialysed horse serum. One important observation from these studies was the differences in the concentration of specific nutrients required for optimal growth of HeLa (Gey et. al. 1952) and mouse L cells (Sanford et. al. 1948). Today, a myriad of defined growth media, each catering to the specific nutritional

requirements of various cell types are used. However these are all similar to the original synthetic media formulations (Eagle 1955a, 1955b).

Another major development in mammalian cell culture followed the introduction of the use of chemically defined growth media. Up to 1960, only limited success was achieved in propagating diploid primary cells with a stable genomic configuration for extended periods of time. The importance of the establishment of such cell cultures is two-fold. Firstly, a stable diploid karyotype will allow such cells to be used for precise genetic studies and secondly the objection to the use of heteroploid cell lines displaying similar phenotypic properties of malignant cells (Hayflick and Moorhead 1961) for vaccine production could be circumvented. Hayflick and Moorhead (1961) first reported the isolation and characterization of numerous strains of human fetal fibroblasts which were amenable for culture and vaccine production. These cell strains, besides having a stable diploid karyotype, also had growth and morphological characteristics of normal primary cell strains.

The advent and refinement of tissue culture techniques have proven to be an invaluable tool for probing the biochemical and molecular basis for human

diseases and the prenatal diagnosis of fetuses which may have various genetic related illnesses. And now, with the introduction of cell hybridization, chromosome mapping and genetic cloning techniques, tissue culture will continue to be an exciting and fruitful field for research.

(b) Growth Requirements and Characteristics of Cultured Mammalian Cells

With improvement in cell culture techniques, cell culturists have, in recent years, increasingly focused their attention on the control of mammalian cell growth and division by serum macromolecular factors and low molecular weight nutrients. Much of the data suggest that normal mammalian cellular processes are tightly controlled by a complex interaction of such growth factors and simple nutrients (Holley 1975; Gospodarowicz and Moran 1976). All cell lines studied have shown that serum is an absolute growth requirement. This led to early attempts to isolate specific growth factors from serum. The first macromolecular serum fraction isolated was fetuin (Lieberman and Ove 1957;Fisher et. al. 1958) and it was shown to be essential for promoting cell attachment and stretching on a glass substratum. Since then,

numerous growth factors have been isolated from serum, various mammalian tissues and even from established cultured mammalian cells (Gospodarowicz and Moran 1976). Recent publications (Rizzino and Sato 1978; Hayashi and Sato 1976) reported the successful growth of various cell lines in serum free media which had been supplemented with purified fetuin, numerous hormones, transferrin and 2-mercaptoethanol. These studies by Sato and coworkers (Rizzino and Sato 1978; Hayashi and Sato 1976) and Gospodarowicz and Moran (1976) strongly suggest that one of the major roles of serum in cell culture is for the supply of hormones.

Accumulated data for the effects of low molecular weight nutrients on the growth of cell lines are extensive. However, at least for mouse L cells (Sanford et. al. 1948) and HeLa cells (Gey et. al. 1952), the essential nutrilites are D-glucose, L-arginine, L-cysteine, L-glutamine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine and L-valine. Of all the amino acids supplied, L-glutamine is required at the highest concentrations as cell growth is rapidly arrested on removal or depletion of it (Eagle et. al. 1955; Ley and Tobey 1970; Pardee 1974). Glutamine is an important

metabolite considering its involvement in protein biosynthesis, de novo synthesis of nucleotides, as an energy source, synthesis of coenzymes and in several aminating reactions (Stadtman 1973; Zielke et. al. 1976). Vitamins, cofactors and ions such as choline, folic acid, nicotinamide, pantothenic acid, pyridoxal, riboflavin, thiamine, Na^{+}, K^{+}, Mg^{++}, Ca^{++}, Cl^{-} and PO_4^{3-} are also essential (Eagle 1955c). Trace elements and other vitamins may be supplied by the addition of serum. The unique physiological roles of some of the ions in mammalian tissues was first recognized by Ringer in 1882. It is obvious from studies that most of the physiological processes require some ions as cofactors. Calcium, particularly its role in the cAMP response, is of paramount importance in the regulation of cell growth (Rasmussen 1970). Of interest are two recent reports (Rubin and Sanue 1977; Barnes and Colowick 1977) which demonstrated that extracellular complexes of Ca^{++}, PPi and HPO_4^{2-} are involved in the stimulation of cellular proliferation, sugar uptake and thymidine incorporation in mouse 3T3 cells.

Transformed cells in culture have been shown to have different morphology and growth requirements from normal primary cell lines. Transformed cells generally

have a lower requirement for the macromolecular serum factors for growth (Temin 1966). In certain instances, different fractions of serum stimulate growth of normal and transformed cells (Paul et. al. 1971; Rudland et. al. 1974). Also transformed cells continue to proliferate at suboptimal nutrient conditions unlike normal untransformed cells (Holley 1975) and will eventually die if fresh medium is not supplied. This has led to a postulation that transformed cells may have lost their so called restriction point (R-point) control (Pardee 1974). This has some supportive evidence in that, in vivo, tumour cells require an ample supply of nutrients to survive (Folkman et. al. 1974). Some of the other characteristics expressed in culture by transformed cells as compared to normal cells are an unlimited proliferative lifespan (Hayflick and Moorhead 1961), drastic karyotypic alterations (Levan and Biesele 1958; Todaro And Green 1963; Hayflick And Moorhead 1961), ability to grow in suspension culture (Macpherson and Montagnier 1964), disoriented growth patterns (Stoker and Abel 1962; Temin and Rubin 1958), poor attachment and spreading ability on a solid substrate (Abercrombie 1970; Ambrose et. al. 1970) possibly due to changes in the size and number of actin cables (Tucker et. al. 1978), different composition of

the proteins and morphology of the plasma membrane (Hynes 1976; Robbins and Nicolson 1975), low intracellular levels of cAMP when grown to high densities (Otten et. al. 1971), the agglutinability by wheat germ agglutinin (Aub et. al. 1965; Burger and Goldberg 1967), and the ability to produce tumours when inoculated into appropriate animals (Earle and Nettleship 1943). The better understanding of such _in vitro_ properties of transformed cells in culture, hopefully, may shed some light on the mechanisms involved in _in vivo_ tumourigenesis.

(2) Mammalian Cells: RNA Biosynthesis and Amino Acid Withdrawal

In bacteria, various cellular processes including the accumulation of ribosomal-RNA are abruptly curtailed on starvation for an essential amino acid (Cashel and Gallant 1974; see 'The Stringent Response' in this 'Historical'). Unlike this well-studied 'stringent response' of bacterial cells expressed during amino acid deprivation (Stent and Brenner 1961; Cashel and Gallant 1974), the physiological consequences of amino acid withdrawal from the growth media of cultured mammalian cells remain obscure. For example, the total RNA synthesis

in amino acid starved mouse L-cells and Landschutz ascites tumour cells was unimpaired (Skold and Zetterberg 1969; Shields and Korner 1970). Other reports, however, have shown that deprivation of other mammalian cell lines of essential amino acids led to a decrease either in uridine incorporation into RNA (Bolcsfoldi et. al. 1971; Smulson and Thomas 1969) or the synthesis of pre-rRNA (Vaughan 1972; Jolicoeur and Labrie 1974; Grummt et. al. 1976) and r-RNA (Nakashima et. al. 1976). Concommitantly, mRNA synthesis was not affected (Jolicoeur and Labrie 1974; Nakashima et. al. 1976).

The biochemical mechanisms underlying this regulation of rRNA synthesis, however, are in general unknown. Grummt and Grummt (1976) proposed that rRNA synthesis during amino acid deprivation is directly controlled by the actual concentration of ATP and GTP. These nucleotides have been found to be depleted upon amino acid withdrawal. Recently, a similar study using a different cell line failed to show any significant changes in ATP and GTP pool sizes before and after amino acid withdrawal even though rRNA synthesis was severely limited (Dehlinger et. al. 1977). Other proposed control mechanisms for rRNA synthesis include the requirement of a rapidly turning-over protein factor which is not

produced during amino acid starvation (Cereghini and Franz-Fernandez 1976), or the production of a regulatory molecule, analogous to bacterial ppGpp,during amino acid deprivation which may directly interact with the transcription machinery (Goldberg and St. John 1976). Concrete information on this aspect of mammalian cell growth regulation is therefore rudimentary.

(3) Physiology, Biochemistry and Possible Role(s) of Polyphosphates and Unique Phosphorylated Nucleosides in Procaryotic and Eucaryotic Cellular Regulation

The relationship between all living cells and the external milieu is essentially one of either survival or death. Thus it is essential that cells must have evolved certain intrinsic regulatory mechanisms with which they could rapidly adjust their cellular metabolism according to the prevailing growth environment.The isolation and characterization of various types of polyphosphates and unique phosphorylated nucleosides produced during altered growth conditions have led to the current interest in their possible role as mediators of cellular regulation of growth in procaryotes and eucaryotes. The

following is a historical overview of some of these polyphosphates and unique phosphorylated nucleosides which have been found thus far in both procaryotes and eucaryotes.

(a) Polyphosphates (PolyP)

Polyphosphates (polyP) were first isolated and characterized from yeast by Wiame (1947; 1948) and Schmidt et. al. (1946). Since then, polyP have been isolated from a large number of microorganisms, higher plants and animals (Harold 1966). It has been observed that there is a considerable heterogeneity in the size and quantity of endogenous polyP in different organisms (Harold 1966). On the contrary, PolyP with chain lengths of 3,4,10 and 300 have been isolated from Saccharomyces cerevisiae (Harold 1966; Ludwig et. al. 1977).

The biosynthesis of polyP requires only one reaction that is catalysed by polyphosphate kinase (Harold 1966). This enzyme which was originally found in yeast (Yoshida and Yamataka 1953) and later purified from Escherichia coli (Kornberg et. al. 1956), catalyses the reaction

$$ATP + (Pi)n \longrightarrow ADP + (Pi)n+1$$

It is Mg^{2+} dependent and is strongly inhibited by NaF

and ADP (Kornberg et. al. 1956). The inhibition of the reaction by ADP suggested that a low *in vivo* $^{ATP}/_{ADP}$ ratio would result in a reversal of the reaction.

Unlike the biosynthesis process, the catabolism of polyP is catalysed by numerous enzymes. Presumably, a low cellular $^{ATP}/_{ADP}$ ratio will result in degradation of polyP by the reversal of the polyphosphate kinase reaction. Another degradative enzyme isolated was polyphosphate adenosinemonophosphate phosphotransferase. The purified enzyme (Dirheimer and Ebel 1965) requires Mg^{2+} and catalyses the transfer of phosphate from fairly high chain length polyP to AMP. Szymona (1962a) and Szymona et. al. (1962b) were the first to discover polyphosphate glucokinase which utilizes polyP to phosphorylate glucose to glucose-6-phosphate. This Mg^{2+} dependent enzyme could also use glucosamine but not mannose or fructose as substrates. PolyP glucokinase is present in numerous mycobacteria and in *Corynebacterium diphtheriae* but is absent in *Escherichia coli*, *Aspergillus aerogenes* and *Aspergillus niger* (Szymona et. al. 1962b). Polyphosphatases are apparently heterogeneous and widespread in various microorganisms and animal tissues. In *Saccharomyces cerevisiae*, polyphosphatases that are specific for short chain polyP were found (Harold 1966),

while those purified from Corynebacterium xerosis are specific for high molecular weight polyP. Also no short chain polyP intermediates were detected after hydrolysis suggesting that the enzymatic attack proceeded sequentially from the terminal ends (Szymona et. al. 1962b). In contrast, the polyphosphatase from Aspergillus niger cleaved polyP into smaller fragments without liberation of inorganic phosphate (Sall et. al. 1956). Finally a rather unique polyP degradative enzyme, polyphosphate fructokinase, was isolated from Mycobacterium phlei (Szymona and Szumilo 1966). This enzyme catalyses the transfer of Pi from polyP and ATP to fructose. The biosynthesis of this enzyme is inhibited by glucose and induced in fructose grown cells.

In spite of the accumulation of data on polyP metabolism, little is known about its biological functions. Physiological studies, however, related to polyP metabolism have shed some light on its significance.

A common feature of polyP metabolism is the large fluctuation in intracellular pool sizes which itself is dependent on the growth conditions. PolyP levels are low in organisms undergoing rapid growth while elevated levels prevail during conditions of nutritional imbalance and thus resulting in growth inhibition (Harold 1966). In

bacteria, polyP accumulation is triggered by nitrogen (Sall et. al. 1956), sulfur (Harold 1966) and zinc (Winder and O'Hara 1962) starvation. Related to nitrogen starvation was the inverse relationship observed between nucleic acid biosynthesis and polyP levels in Aerobacter aerogenes (Harold 1966). Owing to the fact that upon resumption of growth and the concommitant degradation of polyP there is incorporation of the released Pi into nucleic acids, it has been suggested (Harold 1966) that polyP may act as a phosphate storage sink during periods of non-growth. In yeast and filamentous fungi, similar physiological properties have been observed. The involvement of polyP in growth and differentiation of Aspergillus niger and Physarum polycephalum is well documented (Nishi 1961; Goodman et. al. 1969). In Aspergillus niger, large amounts of polyP are found in the spores. During the germination process polyP was found degraded by elevated levels of polyphosphatases to Pi thus serving as a source for phospholipid and nucleic acid synthesis (Nishi 1960; Nishi 1961). Goodman et. al. (1969) reported that in Physarum polycephalum the level of insoluble polyP was low during periods of active RNA and DNA synthesis and high during the differentiation (encystment) process. Hildebrandt and Sauer (1977) proposed

that insoluble polyP may be involved in the differentiation process of this organism by specifically inhibiting rRNA synthesis.

Obviously the existence of huge amounts of polyP in so many diverse organisms and plants points to an important role for it in cellular regulation. Data to ascribe a definitive role(s) for polyP, however, remain elusive.

(b) Adenosine 3':5' Cyclic Monophosphate (cAMP)

(i) Cyclic AMP: Biosynthesis, Catabolism and Possible Regulatory Role(s)

Cyclic AMP was first isolated and characterized by Sutherland and Rall (1958). The synthesis of cAMP from ATP is catalysed by a plasma membrane located enzyme, adenylate cyclase. The activity of this enzyme is dependent on a divalent cation (Mg^{2+}or Mn^{2+}) but is strongly inhibited by Ca^{2+} (Robison et. al. 1971).

The catabolism of cAMP to 5'AMP is effected by a cAMP phosphodiesterase which was first found in heart, brain and liver extracts (Sutherland and Rall 1958). Like adenylate cyclase, this phosphodiesterase is also

Mg^{2+} dependent and its activity is strongly inhibited by the methylxanthines, theophylline and caffeine (Robison et. al. 1971).

The implication of cAMP as a possible regulatory molecule stems from observations that certain physiological responses resulting from hormonal stimulation were interposed by transient elevation of intracellular cAMP levels (see Robison et. al. 1971). The best understood of this phenomenon is the action of epinephrine and glucagon on glycogenolysis. Data show that upon hormonal stimulation of specific tissues, cAMP levels become elevated (Sutherland and Rall 1958). This cyclic nucleotide then activates a specific cAMP-dependent protein kinase which in turn activates phosphorylase b kinase through direct phosphorylation of the kinase (Walsh et. al. 1968; Robison et. al. 1971), thus ultimately leading to glycogen catabolism. Numerous cAMP-dependent protein kinases have since been found in various mammalian tissues and other eucaryotes (see Robison et. al. 1971 and Rosen et. al. 1977). It should be pointed out that the two existing classes of cAMP-dependent protein kinases are themselves regulated in a complex way by a heat and acid stable inhibitory protein found in numerous mammalian tissues (Rosen et. al. 1977). Recent studies have found other protein kinases

that are cAMP independent (Rosen et. al. 1977). However little is known about the regulation of these proteins. It is pertinent to point out here that numerous hormonal actions are independent of cAMP metabolism (Robison et. al. 1971).

Cyclic AMP has now been detected in bacteria, various unicellular organisms, most animal tissues and in some plants (Rickenberg 1974; Robison et. al. 1971). However owing to the voluminous amount of data, only some of the salient aspects of cAMP metabolism and function(s) will be discussed in the following sections.

(ii) Cyclic AMP in Bacteria

When bacteria are cultured under glucose-rich conditions, the synthesis of various inducible enzymes necessary for the utilization of other sugars are either repressed or occur at greatly reduced rates even in the presence of the specific inducing sugar. The bacteria will synthesize the enzymes required for the metabolism of other energy-yielding sugars only when the glucose supply is exhausted (Monod 1947). This intriguing metabolic feature of bacteria is known as catabolite repression (Magasanik 1961).

Makman and Sutherland (1965) were the first to discover that glucose could lower cAMP levels in E. coli. This led to a series of elegant studies which provided an insight into one of the functions of cAMP in procaryotes. Pastan and Perlman (1970) demonstrated that in the presence of both glucose and lactose, high levels of exogenously supplied cAMP could overcome glucose-mediated repression of the synthesis of β-galactosidase and other inducible enzymes. The subsequent isolation of appropriate bacterial mutants proved invaluable in these studies. For example, adenylate cyclase deficient E. coli were unable to ferment various sugars even in the absence of glucose unless cAMP was provided. Revertants of such mutants were invariably found to possess normal adenylate cyclase activity (Perlman and Pastan 1969). Another group of mutants failed to metabolize various sugars even in the presence of cAMP. The lesion was subsequently found to be due to an absence of a cAMP binding protein known as either the catabolite gene activator protein (CAP) or the cAMP receptor protein (CRP) (Emmer et. al. 1970; Zubay et. al. 1970). Using a cell-free in vitro transcription system, de Crombrugghe et. al. (1971) showed that the synthesis of lac messenger RNA required both cAMP and CRP. Pastan and his collaborators (Pastan

and Perlman 1972; de Crombrugghe et. al. 1971) have proposed a model for the mechanism of cAMP-CRP action on the lac transcription system. Cyclic AMP first binds to CRP to form a complex which in turn binds to a site on the promoter of the lac operon. This then facilitates the effective binding of RNA polymerase to another promoter region leading to the initiation of transcription. There is genetic evidence to suggest that these two postulated promoter sites exist (Beckwith et. al. 1972). Thus the rate of transcription may be regulated by the relative amounts of intracellular cAMP which in turn is dependent on the external source of glucose in the growth environment. This phenomenon clearly demonstrates the important second 'messenger' (Robison et. al. 1971) role of cAMP in bacteria.

(iii) Cyclic AMP in the Fungi

Since the demonstration of cAMP in various fungi (see Robison et. al. 1971), the possible involvement of this cyclic nucleotide in the metabolism of such organisms has been reported. The classical organism used in most of these studies is the cellular slime mould, Dictyostelium discoideum. In the vegetative phase, the organism occurs as a unicellular amoeba. On starvation,

the amoeboid cells aggregate into multicellular structures which further differentiate into fruiting bodies.

The aggregation phenomenon due to starvation is unique in that initially, only a few amoeba actually release cAMP. These cells form the aggregation nucleus, which in turn excretes cAMP in pulses (Bonner 1971) thus attracting other nearby amoebae to form larger aggregation centres. This aggregation process is also specifically stimulated by exogenously added cAMP (Konijn et. al. 1967). The role of a cAMP phosphodiesterase in regulating the cyclic nucleotide levels and thus the aggregation process has been suggested by Riedel and Gerisch (1971). D. discoideum amoebae apparently excrete a cAMP phosphodiesterase during vegetative growth. Under unfavourable growth conditons, however, just prior to aggregation, an inhibitor of this enzyme is released into the medium (Riedel et. al. 1973). The lack of this cAMP phosphodiesterse inhibitor in non-aggregating mutants suggested that the development phase of D. discoideum is initiated, in part, by changes in the extracellular levels of cAMP (Riedel and Gerisch 1971). In addition to the aggregation role, cAMP added at high concentrations to amoeboid cells caused them to

differentiate only into stalk cells (Bonner 1970).

Other effects of cAMP in fungi include the cell cycle-dependent inhibition of a protein kinase activity in Physarum polycephalum (Kuehn 1972) and the reversal of glucose repression on sporulation in yeast (Tsuboi et. al. 1972). Also cAMP may be involved in glycogenolysis in Neurospora crassa (Tellez-Inon and Torres 1970), the regulation of citrate fermentation and sucrose utilization by Aspergillus niger (Wold 1974) and during the germination process of Blastocladiella emersonii (Gomes et. al. 1978).

(iv) Cyclic AMP in Mammalian Cells in Culture

The most interesting studies on cAMP metabolism have been those related to its role in the regulation of growth of mammalian cells in culture. Burk reported that growth was impeded by the addition of cAMP phosphodiesterase inhibitors to normal and transformed baby hamster kidney cells (Burk 1968). Subsequently, Ryan and Heidrick showed the direct inhibitory growth effect of cAMP on cultured cells (Ryan and Heidrich 1968). Various cell lines derived from different tissues are now known to have a decreased growth rate in the presence

of added cAMP (or cAMP analogues) or various agents which raise intracellular cAMP levels (Pastan et. al. 1975). Further support for the role of cAMP as a physiological growth inhibitor was provided by Pastan and co-workers. Johnson and Pastan reported that prostaglandin E_1 slowed the growth of cultured fibroblasts. This agent which is known to activate adenylate cyclase and therefore raises intracellular cAMP was found to be ineffective in inhibiting growth of a mutant resistant to prostaglandin E_1 activation. Also prostaglandin B_1, which does not elevate intracellular cAMP was not growth inhibitory (Johnson and Pastan 1971; Johnson et. al. 1972). Cholera toxin, which has been found to inhibit DNA synthesis and therefore growth, probably acts like prostaglandin E_1 in being able to activate adenylate cyclase (Hollenberg and Cuatrecases 1973). Serum deprivation of normal cells results in growth inhibition at the G_1 phase of the cell cycle and with the concommitant elevation of intracellular cAMP (Pardee 1974; Kram et. al. 1973). On the other hand, the effect of nutrient starvation on cellular cAMP remains unclear. Jolicoeur et. al. (1974) reported that cAMP concentration in Landschutz tumour cells was unperturbed by total amino acid starvation while cAMP accumulated in Balb 3T3 cells deprived of glutamine and histidine (Pastan et. al. 1975).

This discrepency may be due to physiological differences between the cell lines examined. Besides being a possible growth regulator, cAMP has also been implicated in affecting the differentiated state of cells in culture (Baum et. al. 1978; Miller et. al. 1978)

One of the characteristics of transformed cells in culture, unlike normal cells, is their ability to continue to grow beyond confluency and to maintain low levels of cAMP under such conditions (Otten et. al. 1971). Carchman et. al. (1974) demonstrated the relationship between the transformed and normal state of NRK cells with regard to intracellular cAMP levels by infecting such cells with a temperature sensitive Kirsten sarcoma virus. Tomkins and his collaborators have suggested that the 'pleiotypic program' (Hershko et. al. 1971) in mammalian cells is regulated by cAMP, the 'pleiotypic regulator'. Further, they have suggested that the transformed state may be the result of a loss in the control of cAMP metabolism or in the action of this effector (Kram et. al. 1973).

(c) Guanosine 3':5' -Cyclic Monophosphate (cGMP)

Cyclic GMP was originally discovered in 1963 in rat urine (Ashman et. al. 1963). Subsequently, it was

detected in various animal tissues. Guanylate cyclase, the cGMP biosynthetic enzyme, has since been found in all mammalian tissues examined other than sperm. The enzyme is also present in insects, bacteria, birds, fish, fungi and probably in plants. Phosphodiesterases specific for cGMP degradation have also been found (Goldberg and Haddox 1977). In comparison cGMP, like cAMP, is able to activate numerous cGMP specific protein kinases (Kuo and Greengard 1970; Nishiyama et. al. 1975). The mechanism of action (Lincoln et. al. 1977) and the physiological significance of the phosphorylation of specific proteins by these cGMP protein kinases remain unclear. In contrast, cGMP appears to have an antagonistic regulatory effect on those bidirectionally controlled functions which are also mediated by cAMP. This phenomenon is also known as the 'Yin - Yang' effect (Goldberg et. al. 1974). For example during the cell cycle of Novikoff hepatoma cells, high extracellular levels of cGMP were found during mitosis with correspondingly low concentrations of cAMP (Zeilly and Goldberg 1977). Other 'Yin - Yang' regulated biological functions include cardiac muscle contraction, lysosomal secretion by leucocytes, neuronal excitability, lymphocyte proliferation, response of polymorphonuclear leucocytes to chemotactic stimuli and T-lymphocyte mediated

cytotoxicity (see Goldberg and Haddox 1977).

Another significant difference between cGMP and cAMP metabolism is the lack of evidence to show that agents which stimulate the accumulation of intracellular cGMP also activate guanylate cyclase activity *in vitro* (Goldberg and Haddox 1977) unlike that for cAMP metabolism (Robison et. al. 1971). This is further substantiated by the fact that guanylate cyclase is relatively insensitive to hormonal activation (Goldberg and Haddox 1977). These differences suggest that cGMP functions in an unique way; a role(s) which has yet to be unravelled.

(d) Bacterial MS (ppGpp and pppGpp) Nucleotides

(i) The Stringent Response

In bacteria, at least, the deprivation of a growth essential amino acid or the limitation of transfer RNA (t-RNA) aminoacylation will result in an abrupt cessation of numerous cellular activities commonly known as the 'stringent response' (Stent and Brenner 1961). Among the cellular activities curtailed are the accumulation of stable RNA (Cashel and Gallant 1974), biosynthesis of lipids (Sokawa et. al. 1968), nucleotides (Gallant et. al.

1971), polyamines (Holffa et. al. 1974), uptake of purines and pyrimidines (Nierlich 1968; Edlin and Neuhard 1968), phosphate (Cashel and Gallant 1968), glucose (Sokawa and Kaziro 1969) and an increase in cellular proteolysis (Sussman and Gilvarg 1969).

Bacterial mutants which failed to restrict RNA accumulation during amino acid starvation and are therefore said to possess 'relaxed' control of RNA synthesis were subsequently isolated (Borek et. al. 1955; Stent and Brenner 1961). This anomaly was the result of a mutation at a genetic locus called the RC gene (Stent and Brenner 1961), which has since been renamed the relA gene. Thus the wild type bacterial stringent strain bears the relA$^+$ allele, while the 'relaxed' strain the relA$^-$ allele (Fiil and Friesen 1968). Fiil (1969) demonstrated that the relA$^+$ gene product is the cytoplasmic mediator of RNA accumulation. The role of the relA$^+$gene product is discussed in the next section.

(ii) In Vivo Synthesis of ppGpp and pppGpp

Owing to the observation that many diverse physiological effects of amino acid starvation in bacteria (as described in preceding section) are not the direct result of blocked RNA accumulation (Cashel and Gallant 1968;

Gallant and Harada 1969), it was proposed by Cashel and Gallant (1968) that the function of the relA gene product was essential for the synthesis of a metabolic inhibitor. Subsequently two unusual nucleotides, 'magic spot' I and II (MSI and MSII),were isolated but only from acid extracts of amino acid starved relA$^+$ E. coli and not from relA$^-$ cells (Cashel 1969; Cashel and Gallant 1969).

Lund and Kjeldgaard (1972) have estimated that levels of ppGpp, accumulated under stringent conditions, are about the same as intracellular ATP concentrations. Using other E. coli strains (Cashel 1975; Gallant and Harada 1969), it has been estimated that during amino acid deprivation the cellular ATP concentration is 10-12 mM, while the GTP level drops to 2 mM from 4 mM. During this period, ppGpp normally rises to about 4 mM. pppGpp is typically about one-third that of ppGpp though its been reported that the accumulation of pppGpp is higher than ppGpp in Bacillus subtilis (Swanton and Edlin 1972). There are exceptions whereby relA$^+$ bacterial strains did not accumulate pppGpp under stringent conditions (Cashel 1969;discussed later).

Chemical analyses of purified ppGpp showed it to be guanosine-5'-diphosphate-3'-diphosphate (Cashel and Kalbacher 1970; Sy and Lipmann 1973) while pppGpp is

probably guanosine-5'-triphosphate-3'-diphosphate (Haseltine et. al. 1972).

Studies of the kinetics of ppGpp metabolism suggested that this unique molecule may be involved in blocking RNA accumulation during the 'stringent response'. For example, when RNA synthesis was directly inhibited with antibiotics, no ppGpp was formed; thus implying that ppGpp production is not a secondary consequence of blocked RNA synthesis (Cashel and Gallant 1969; Gallant et. al 1970; Lazzarini et. al 1971). Also ppGpp accumulation commences within seconds on amino acid deprivation while the inhibition of RNA synthesis is not apparent until a minute later (Cashel 1969; Gallant et. al. 1970; Fiil et. al. 1972). On reversal of the stringent state, the recovery of RNA synthesis is preceded by a rapid first-order decay of the accumulated ppGpp (Cashel 1969; Stamminger and Lazzarini 1974).

Another feature of ppGpp metabolism is the restoration of RNA synthesis and the absence of ppGpp when protein synthesis inhibitors were added to amino acid starved relA$^+$ cells (Cashel 1969; Lund and Kjeldgaard 1972). This result was simply explained by the trickle-charging of the t-RNA species specific for the missing amino acid by free amino acids generated during intracellular protein turnover when protein synthesis

was totally inhibited (Kurland and Maaloe 1962). Kaplan et. al.(1973) have reported, however, that using temperature sensitive t-RNA charging mutants, both ppGpp and pppGpp accumulation were inhibited by tetracycline even when one species of t-RNA was completely deaminoacylated. These data suggest that the presence of both deacylated t-RNA and an intact protein synthesizing apparatus are necessary for relA$^+$ bacteria to exhibit the stringent state and for the generation of ppGpp and pppGpp (Cashel 1975). This agrees with the proposal that these unique guanosine nucleotides are produced by an 'idling reaction' of protein synthesis (Cashel and Gallant 1969). The inhibitory effect of rifampicin, an mRNA synthesis inhibitor, on ppGpp synthesis during amino acid deprivation is unknown. It is possible that mRNA is required for the 'idling reaction' and thus for ppGpp synthesis (Wong and Nazar 1970; de Boer et. al. 1973). The data available, therefore suggest that the relA gene product is a factor associated with the bacterial protein synthetic apparatus.

Recently another genetic locus, designated spoT for 'spotless', has been implicated in the bacterial stringent response. The gene product of the spoT locus is probably involved in the conversion of ppGpp to pppGpp as evidenced by the following in vivo observations.

Firstly certain E. coli spoT$^-$ strains failed to accumulate any appreciable levels of pppGpp during amino acid starvation even though intracellular concentrations of ppGpp were nearly triple those attained by spoT$^+$ strains under identical growth conditions (Laffler and Gallant 1974). Secondly, when spoT$^-$ mutants were replenished with the missing amino acid, the decay of ppGpp was approximately ten to thirty fold slower than for spoT$^+$ strains (Laffler and Gallant 1974; Stamminger and Lazzarini 1974). This possible mechanism of ppGpp and pppGpp metabolism in spoT bacterial strains has yet to be demonstrated in in vitro experiments. But recent in vivo and in vitro studies (Chaloner-Larsson and Yamazaki 1976; Fiil et. al. 1977; Wyer et. al. 1976; Heinemeyer et. al. 1978) have resulted in the postulation of a different model for the consequences of a mutation at the spoT locus (Sy 1977). The model encompasses the following salient features. Firstly, the primary result of the spoT mutation is a reduction in the rate of ppGpp catabolism to GDP. Secondly, pppGpp is the precursor of ppGpp and not vice versa as suggested by Laffler and Gallant (1974). Thirdly, ppGpp negatively controls the conversion of GTP to pppGpp and fourthly, a catabolite of ppGpp negatively controls the conversion of pppGpp to ppGpp. An important point to note is that these stringent spoT$^-$ E. coli

behave exactly like stringent *spoT*$^+$ cells with regard to the reciprocal relationship between rates of RNA synthesis and the levels of intracellular ppGpp (Cashel 1969; Lund and Kjeldgaard 1972; Stamminger and Lazzarini 1974).

(iii) *In Vitro* Synthesis of ppGpp and pppGpp

The synthesis of ppGpp and pppGpp using an *in vitro* *E. coli* system was first reported by Haseltine et. al. (1972). GDP and GTP were the required substrates for ppGpp and pppGpp synthesis respectively with ATP being the pyrophosphate donor. Other requirements for this synthesizing system, besides buffers and salts, are high salt washed ribosomes and a so called 'stringent factor' (Haseltine et. al. 1972) present in the 0.5 M NH_4Cl wash of ribosomes. When a full complement of amino acids was added to the ppGpp-pppGpp synthesizing mixture, no ppGpp or pppGpp were made with the high salt washes from the ribosomes of either stringent or relaxed bacterial strains. In the absence of added amino acids, however, only the synthesizing assay containing the 'stringent factor' from stringent strains was able to produce considerable amounts of ppGpp and

pppGpp. This is in agreement with the *in vivo* synthesis of these unique guanosine nucleotides (Cashel 1969; Cashel and Gallant 1969). *In vitro* mixing experiments showed that without added amino acids, ppGpp and pppGpp were synthesized even when both high salt ribosomal washes from stringent and relaxed strains were present (Block and Haseltine 1973). This confirmed the genetic studies which showed that the *relA*$^{+}$ allele is dominant (Fiil 1969). It is of interest to note that all independent *relA*$^{-}$ mutants have been found to produce some residual 'stringent factor' activity as detected by the *in vitro* ppGpp-pppGpp synthesizing assay. The low activity from each mutant was directly related to its characteristic thermolability (Block and Haseltine 1973).

Other studies, using highly purified 70S ribosomes, indicated that both mRNA and t-RNA were also required for the *in vitro* synthesis of bacterial ppGpp and pppGpp (Haseltine and Block 1973; Pederson et. al. 1973), thus suggesting that the original ribosomal preparations of Haseltine et. al. (1972) were actually contaminated with these two RNA species. Using a highly purified modification of the Haseltine et. al. (1972) procedure, Pederson and coworkers (1973)

and Haseltine and Block (1973) were able to demonstrate that maximum rates of ppGpp and pppGpp synthesis could be achieved in the presence of a complete protein synthesis initiation complex with an uncharged t-RNA binding to its specific codon situated at the acceptor site. This mechanism of guanosine tetra- and pentaphosphate synthesis is similar to that previously proposed by Cashel and Gallant (1969).

(iv) Effects of ppGpp and pppGpp on Cellular Enzymes, Translation and Transcription In Vitro

Numerous in vivo and in vitro studies suggest that many bacterial cellular processes may be blocked during periods of elevated intracellular ppGpp and pppGpp levels. For example acetyl-CoA carboxylase which is involved in lipid biosynthesis is inhibited by ppGpp (Polakis et. al. 1973). The de novo biosynthesis of both AMP and GMP from IMP can be curtailed by ppGpp through the inhibition of adenylosuccinate synthetase and IMP dehydrogenase respectively (Gallant et. al 1971). Using isolated bacterial membrane vesicles, Hochstadt-Ozer and Cashel (1972) were able to show that ppGpp potently inhibited purine uptake and the activities of various

purine phosphoribosyltransferases. ppGpp and pppGpp, at a concentration of 1 mM, inhibited bacterial ADP-glucose synthetase (a rate limiting enzyme for glycogen synthesis) by 61% and 77% respectively (Dietzler and Leckie 1977).

The effect of ppGpp and pppGpp on bacterial protein synthesis is at present rather ambiguous. In vitro experiments demonstrated that ppGpp, being quite similar in structure to GTP, inhibited protein synthesis by preventing the formation of the protein synthesis initiation complex (Yoshida et. al. 1972). However it is reasonable to assume that in vivo protein synthesis would itself be blocked during amino acid deprivation. The physiological significance of the relationship between ppGpp and protein synthesis remains obscure.

The inverse relationship between rRNA accumulation and ppGpp and pppGpp levels is well documented. Studies using partially purified DNA-dependent RNA polymerases showed that ppGpp specifically inhibited RNA synthesis (Cashel 1970). Subsequent reports by Reiness et. al. (1975) and van Ooyen et. al. (1976) demonstrated that rRNA synthesis was preferentially and significantly inhibited by ppGpp and pppGpp probably through the inhibition of rRNA chain initiation (van Ooyen et. al. 1976; Travers 1973; Travers 1976). The templates used

in the highly purified *in vitro* transcription-translation system were either *E. coli* DNA or phage DNA carrying an *E. coli* rRNA cistron (Reiness et. al. 1975; van Ooyen et. al. 1976). The *E. coli* DNA-dependent *in vitro* synthesis of various ribosomal proteins, protein elongation factors and RNA polymerase subunit α, were also inhibited by ppGpp (Chu et. al. 1976; Lindahl et. al. 1976). The *in vivo* synthesis of protein elongation factors, at least, was previously shown to be under stringent control (Furano and Wittel 1976). Data support the conclusion that these physiological blocks by ppGpp occur at the level of initiation of transcription (Lindahl et. al. 1976).

In contrast to the numerous *in vitro* inhibitory effects of ppGpp and pppGpp, one of these, ppGpp, has a novel feature in that it was found to stimulate the *in vitro* transcription of lactose, arabinose, tryptophan and histidine operons of *E. coli* and *Salmonella typhimurium* (Yang et. al. 1974; Stephens et. al. 1975).

These *in vitro* observations suggest that ppGpp and pppGpp affect bacterial metabolism in complex ways, particularly the transcription process. It has been proposed that ppGpp functions as a general transcriptional effector (Reiness et. al. 1975) through

its binding to the DNA-dependent-RNA polymerase holoenzyme. Depending on how the ppGpp-holoenzyme complex interacts with specific gene/operon promotor sites, ppGpp should have a positive, negative or no effect on the transcription of the gene or operon in question (Yang et. al. 1974). Through this mechanism, it is conceivable that ppGpp and pppGpp are able to coordinate some of the cellular processes so as to provide optimal survival conditions during rapid changes of the nutritional state in bacteria. The isolation and characterization of RNA polymerase mutants and the sequencing of specific genetic promotor sites might lead to a better understanding of this intriguing phenomenon.

(v) Influence of Carbon and Nitrogen Sources on ppGpp, pppGpp and rRNA Metabolism

In contrast to the 'stringent response' due to amino acid deprivation, bacterial cells undergo a differently programmed metabolic response upon a shift from either a rich complex medium to a glucose minimal one or from a rich carbon source to a poorer one. What is unique is that both relaxed and stringent strains were able to shut-off RNA accumulation to varying degrees with the onset of such a nutritional shift

(Maaloe and Kjeldgaard 1966; Lazzarini and Winslow 1970). Concommitantly, both bacterial strains were able to accumulate ppGpp (Lazzarini et. al. 1971; Harshman and Yamazaki 1971; Winslow 1971). The intracellular levels of ppGpp in both relA$^+$ and relA$^-$ downshifted cells, however, were only about 25-50% of those attained by amino acid starved bacteria (Lazzarini et. al. 1971; Harshman and Yamazaki 1971; Winslow 1971). pppGpp concentrations remained either at basal levels or decreased slightly during stepdown conditions (Harshman and Yamazaki 1971), a response similar to that expressed by amino acid starved spoT$^-$ mutants (Laffler and Gallant 1974).

The depression in rRNA accumulation in downshifted cells is the result of a decrease in rRNA synthesis and an increase in rRNA degradation (Erlich 1972; Norris and Koch 1972). The role of ppGpp in inhibiting rRNA synthesis is clear (Reiness et. al. 1975; van Ooyen et. al. 1976). In studies where rRNA levels were found to decrease specifically by degradation during downshift, no ppGpp was produced (Lazzarini et. al. 1969; Erlich et. al. 1975). Thus during downshift, ppGpp specifically affects rRNA synthesis while another mechanism exists for the preferential degradation of newly made rRNA

(Lazzarini et. al. 1969). Gallant and Lazzarini (1976) have hypothesized that specific rRNA nucleases subject to control, possibly by yet to be discovered nucleotides, may be involved in this rRNA degradative process.

(e) HPN Compounds

The regulation of bacterial sporulation has been an intriguing problem for research scientists. Using Bacillus megaterium, Elmerich and Aubert (1973) found that sporulation by certain mutants with lesions in the purine biosynthetic pathway was derepressed when grown in a glucose-ammonia containing medium. They suggested that certain intermediates in the de novo purine biosynthetic pathway are involved in bacterial sporulation, possibly by mediating the synthesis of either a repressing compound or inhibiting the production of an inducing compound.

Rhaese and coworkers (1972; 1976) were the first to report the existence of four acid extractable, highly phosphorylated compounds, HPN I, II, III and IV in the sporulating bacterium, Bacillus subtilis. HPN I, II, III and IV were subsequently reported to be adenosine 3',5'-diphosphate (ppApp), adenosine 3'-

diphosphate 5'-triphosphate (pppApp), uridine 3'-monophosphate 5'-monophosphate-Z-diphosphate (ppZpUp, where Z is an unknown sugar) and adenosine 3',5'-triphosphate (pppAppp) respectively (Rhaese et. al. 1977). HPN I and IV have also been reported to be synthesized *in vitro* using isolated bacterial membrane vesicles with ATP as a substrate (Rhaese and Groscurth 1976). These and other studies by Rhaese et. al. (1976; 1977) led the authors to suggest the possible involvement of some of these HPN compounds in bacterial sporulation. When *B. subtilis* attains the stationary growth phase, presumably due to the depletion of essential growth nutrients, or was starved for nitrogen and phosphate, sporulation would inevitably occur. At the onset of sporulation, HPN III and IV accumulated rapidly within the cell. A mutant which was impaired in HPN IV synthesis was found to be asporogenous. Interestingly, a revertant of this mutant regained both the ability to sporulate and synthesize HPN IV (Rhaese et. al. 1977). But the mechanism(s) by which these HPN compounds regulate sporogenesis remains to be elucidated.

It is now pertinent to point out that the physiology of *B. subtilis* sporulation, particularly

with regard to the time of intracellular accumulation of HPN III and IV and the nutritional growth conditions required for inducing their synthesis exhibits a similarity to the involvement of HS compounds in the sporulation process of the eucaryote, Achlya (see part (i) of this section). Whether these two sets of highly phosphorylated compounds are similar, both in structure and function(s), awaits more rigorous chemical and biochemical studies.

(f) Diguanosine Polyphosphates

Numerous polyphosphorylated nucleosides have been found in various eucaryotes. The earliest of these, P^1-P^4 diguanosine 5'-tetraphosphate (Gp_4G) and P^1-P^3 diguanosine 5'-triphosphate (Gp_3G), were found in high concentrations in the encysted embryo of the brine shrimp, Artemia salina and in Daphnia magna (Finamore and Warner 1963; Warner and Finamore 1965; Oikawa and Smith 1966). Clegg and coworkers (1967) suggested that Gp_4G may be used as a source of guanine and adenine nucleotides for nucleic acid biosynthesis during the development of A. salina from the embryonic to laval stage. This period is marked by an absence of

de novo purine biosynthesis (Clegg et. al. 1967). Recently Renart et. al. (1976) showed that Gp_4G is a potent activator of GMP reductase from A. salina . This may be physiologically significant as this enzyme could be a major pathway through which GMP derived from Gp_4G hydrolysis is converted to AMP (Renart et.al. 1976).

(g) Diadenosine Polyphosphates

A compound similar to Gp_4G, diadenosine 5',5'''-P^1,P^4-tetraphosphate (Ap_4A) has been detected in numerous mammalian cell lines (Rapaport and Zamecnik 1976). The observation of the inverse relationship between Ap_4A levels and the doubling times of cells led Rapaport and Zamecnik (1976) to propose that this molecule may act as a positive 'pleiotypic mediator' as contrasted with the negative 'pleiotypic mediator' role of cAMP (Kram et. al. 1973). A recent report by Grummt (1978) demonstrated that fairly high concentrations of Ap_4A could stimulate DNA synthesis in G_1 arrested but not exponentially growing, permeabilized, baby hamster kidney cells. Whether this molecule acts as a storage form of ATP or as a growth regulator remains

to be elucidated (Rapaport and Zamecnik 1976).

(h) Triadenosine Pentaphosphate

Since the discovery of interferon in 1957 (see review by Friedman 1977), little is known about the mechanism(s) involved in its potent antiviral effect. But with the discovery of a low molecular weight trinucleotide, produced in cells synthesising interferon, that can inhibit cell free protein synthesis, the underlying mechanism of interferon action may soon be better understood (Roberts et. al. 1976). This protein synthesis inhibitor has since been characterized as pppA2'p5'A2'p5'A and given the pseudonym 'two-five A' (Kerr and Brown 1978). The data available suggest that one action of interferon is to induce the biosynthesis of the enzyme that is responsible for synthesising this inhibitor molecule. Double stranded RNA then activates this trinucleotide 'synthetase' to produce 'two-five A' using ATP as the substrate. Its been postulated that this 'two-five A' molecule in turn probably activates a nuclease that degrades cellular mRNA, thereby effectively stopping protein synthesis and thus viral production. The specificity of the activated nuclease remains an interesting question (Roberts et. al. 1976; Sen et. al. 1976;

Hovanessian et. al. 1977; Kerr and Brown 1978). Further studies on the in vitro and in vivo action of this unique trinucleotide may provide a more detailed insight into the complex induction by interferon of the antiviral state in mammalian cells (Friedman 1977).

(i) Dinucleoside Polyphosphates (HS)

Three unique polyphosphorylated nucleosides have been isolated from whole-cell acid extracts of a variety of fungi (LéJohn et. al. 1975). These compounds may also be isolated by osmotic shock treatment of whole cells, thus suggesting that they may be located on the cell membrane (LéJohn et. al. 1978). One of these three compounds, HS3, was subsequently shown to consist of a glutamyl-ADP-sugar moiety which is covalently linked to a UDP-sugar-tetraphosphate (McNaughton et. al. 1978). The other two, HS1 and HS2, which have been partially characterised appear to have two uridines each with twelve and ten phosphates respectively in contrast to the eight present in HS3 (McNaughton et. al. 1978).

Data have been presented by LéJohn et. al. (1978) implicating these HS molecules as regulators

of sporulation and nucleic acid metabolism. For example, it was observed that a rapid intracellular accumulation of all three HS compounds occurred just prior to sporulation. This accelerated accumulation of HS molecules by Achlya could be triggered either by the depletion of phosphate in the growth medium or transferring the cells from a rich growth medium to one devoid of nutrients. These nutritional 'shocks' invariably led to sporulation. However, when the starvation medium, for example, was supplemented with glutamine or various purine and pyrimidine bases and nucleosides, HS accumulation and sporulation were concomitantly aborted. It was therefore suggested (LéJohn et. al. 1978) that the intracellular accumulation of all HS molecules may be a prerequisite for the sporulation process.

The relationship between HS compounds and nucleic acid metabolism during the growth cycle of Achlya is clear. An inverse relationship was observed when the rates of RNA and DNA synthesis were compared to HS synthesis (LéJohn et. al. 1975; LéJohn et. al. 1978). This suggested that these HS molecules may be acting as negative effectors of RNA and DNA synthesis in vivo. In vitro experiments demonstrated that HS3 and HS2,

at physiological concentrations, were equally potent inhibitors of isolated Achlya DNA-dependent RNA polymerases (McNaughton et. al. 1975; LēJohn et. al. 1978). Lewis et. al (1977) have also shown that HS3 and HS2 are strong inhibitors of the isolated Achlya ribonucleotide reductase, an enzyme involved in the conversion of ribonucleoside diphosphates to their respective deoxyribose forms (Larsson and Reichard 1966). The possible involvement of these HS compounds in regulating fungal energy-linked transport systems have also been postulated (Stevenson and LēJohn 1978; Goh and LēJohn 1978). It is thus likely that these polyphosphorylated dinucleosides indeed play important regulatory roles in the growth and development of Achlya.

More recently, one of the fungal HS compounds, HS3, was also detected in numerous cultured mammalian cell lines (Goh and LēJohn 1977). Physiological studies clearly demonstrated that HS3 accumulated only when glutamine was removed from the normal growth medium as isoleucine deprivation was without effect (Goh and LēJohn 1977; Goh et. al. 1977). Owing to the very important role of glutamine in de novo purine biosynthesis (Mahler and Cordes 1971), studies were

performed using various drugs which affect de novo purine biosynthesis and with mammalian cell mutants defective in de novo and salvage pathways for purine nucleotide biosynthesis. The results demonstrated that HS3 accumulation may be due to a lack of either precursors for purine nucleotide biosynthesis or of purine nucleotides themselves (Goh and LēJohn 1977; Goh et. al. 1977). For example a CHO GAT^- mutant, auxotrophic for glycine, adenosine and thymidine, owing to its defective folate metabolism (McBurney and Whitemore 1974) accumulated HS3 only when it was deprived of adenosine. Further more, azaserine and methotrexate, which are analogues of glutamine and folic acid respectively, and which block de novo purine biosynthesis were active in causing the accumulation of HS3 (Goh and LēJohn 1977). Also, HS3 that accumulated during glutamine deprivation was depleted by all exogenously supplied purine and pyrimidine bases and ribosides; the only exception being thymidine (Goh and LēJohn 1977).

During glutamine starvation of CHO WT and CHO GAT^- cells, it was observed that DNA and RNA synthesis were inhibited in vivo (Goh and LēJohn 1977). Studies by Lewis et. al. (1977) showed that both Achlya and mammalian cell HS3 were potent inhibitors of ribonucleotide reductases

from CHO cells. It was subsequently suggested (Goh et. al. 1977) that both DNA and RNA synthesis in mammalian cells may be regulated in a similar fashion as in Achlya (McNaughton et. al. 1975; Lewis et. al. 1976), by HS3 molecules. The relevant details of these studies of HS3 metabolism in mammalian cells will be presented in the 'Results' section of this thesis.

(j) Other Polyphosphorylated Nuclesides

Other unusual phosphorylated nucleosides with possible physiological functions include the GTP-like 'phantom spot' (Gallant 1976) which is produced by E. coli in response to an energy source downshift. The authors proposed that this molecule may mediate the accumulation of RNA. Two other novel nucleotides, designated DSI and DSII, were recently found in E. coli (Loewen 1976). Their physiological function(s) remains to be elucidated.

(4) Mammalian Purine and Pyrimidine Metabolism

Since the discovery of uric acid by Scheele in 1776 and the subsequent isolation of nucleic acids by Miescher and Kossell (Wyngaarden and Kelley 1972), purine and pyrimidine metabolism have become fruitful areas for biochemical research. The *de novo* biosynthetic pathways of purines and pyrimidines are illustrated by figures 1 and 2 respectively.

The regulation of purine and pyrimidine biosynthesis in mammalian cells and tissues is complex. Most of the studies which have been carried out report the biochemical activities of partially purified enzymes. To extrapolate these findings to their functions in intact cells requires great caution. The pitfalls associated with such *in vitro* studies have been discussed (Henderson et. al. 1977). Also new enzymes related to purine and pyrimidine metabolism are still being reported. The following discussion is concerned with those enzymes of the purine and pyrimidine biosynthetic pathways which studies have suggested to be possible important sites of regulation.

(a) Regulation of *De Novo* Purine Biosynthetic Enzymes in Mammalian Cells and Tissues

Figure (1)

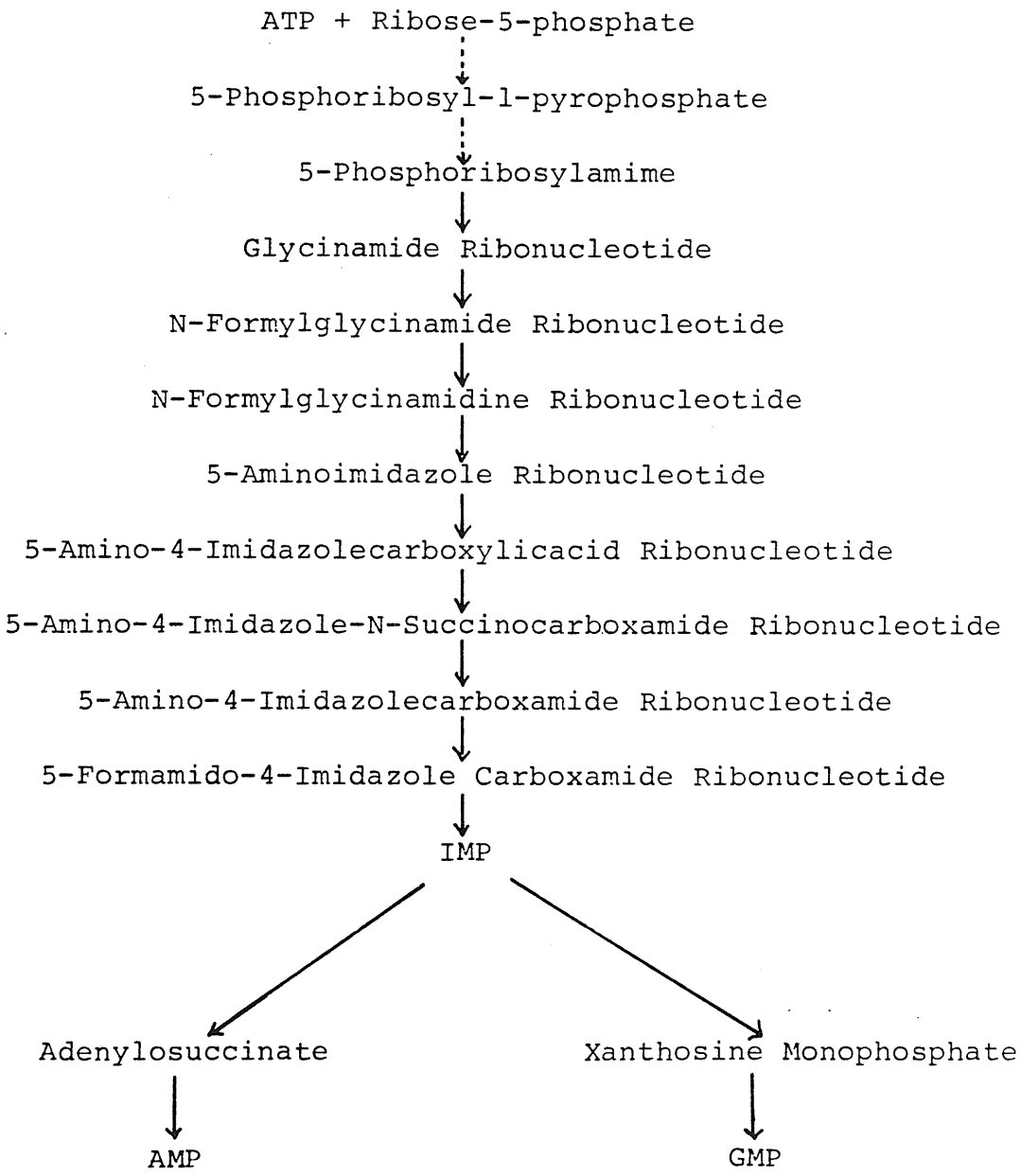
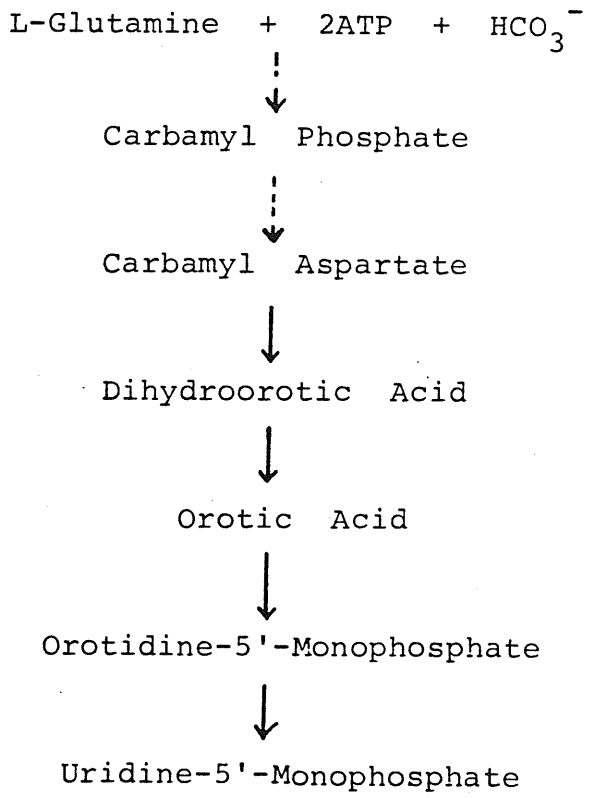

ATP + Ribose-5-phosphate
5-Phosphoribosyl-1-pyrophosphate
5-Phosphoribosylamime
Glycinamide Ribonucleotide
N-Formylglycinamide Ribonucleotide
N-Formylglycinamidine Ribonucleotide
5-Aminoimidazole Ribonucleotide
5-Amino-4-Imidazolecarboxylicacid Ribonucleotide
5-Amino-4-Imidazole-N-Succinocarboxamide Ribonucleotide
5-Amino-4-Imidazolecarboxamide Ribonucleotide
5-Formamido-4-Imidazole Carboxamide Ribonucleotide
IMP
Adenylosuccinate
Xanthosine Monophosphate
AMP
GMP

Figure (2)


L-Glutamine + 2ATP + HCO_3^-
Carbamyl Phosphate
Carbamyl Aspartate
Dihydroorotic Acid
Orotic Acid
Orotidine-5'-Monophosphate
Uridine-5'-Monophosphate

(i) 5-Phosphoribosyl-1-Pyrophosphate Synthetase (PRPP Synthetase) (EC 2.7.6.1) and Intracellular 5-Phosphoribosyl-1-Pyrophosphate Levels

PRPP synthetase, which supplies the necessary cellular PRPP for de novo purine and pyrimidine biosynthesis and purine and pyrimidine salvage reactions (Mahler and Cordes 1971) plays a pivotal role in purine and also pyrimidine metabolism.

PRPP synthetase catalyses the production of PRPP by the following reaction:

$$\text{ribose-5-phosphate} + \text{ATP} \xrightarrow{Pi/Mg^{2+}} \text{PRPP} + \text{AMP}$$

The activity of PRPP synthetase is regulated in a complex manner by magnesium, inorganic phosphate, ATP, ribose-5-phosphate, 2,3-diphosphoglycerate and end-product nucleotides. The enzyme has an absolute requirement for inorganic phosphate, its allosteric activator (Fox and Kelley 1972). This is consistent with the observation that under physiological Pi concentrations, levels of PRPP in human erythrocytes were diminished in the presence of purine nucleosides owing to the lowering of intracellular Pi by the nucleosides (Planet and Fox 1976). The Ehrlich ascites tumour cell's PRPP synthetase, with Mg-ATP being its true substrate, was strongly activated by magnesium

(Murray and Wong 1967). PRPP and 2,3-diphosphoglycerate were found to be competitive inhibitors (Murray and Wong 1967; Fox and Kelly 1972; Hershko et. al. 1969) while the end-product purine nucleotides were non-competitive inhibitors with the nucleoside di- and triphosphates being more potent than the nucleoside monophosphates (Fox and Kelly 1972). These nucleotides regulate partially purified PRPP synthetase by a mechanism described by Fox and Kelley (1972) as 'heterogenous metabolic pool inhibition'. The degree of inhibiton being dependent on the absolute concentration of each of the purine nucleotides in the total cellular nucleotide pool. This mechanism of inhibition has also been observed in vivo (Bagnara et. al. 1974).

Certain PRPP synthetase mutant cells associated with an accelerated rate in PRPP and purine synthesis have recently been described (Becker et. al. 1973; Zoref et. al. 1975). A similar condition exists in tissues and cells deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) (EC 2.4.2.8) (Seegmiller et. al. 1967; Rosenbloom et. al. 1968). Studies using an HGPRT-deficient cultured rat hepatoma cell line suggest that the cause for purine overproduction appears to be the result of an elevated PRPP synthetase activity rather

that a PRPP 'sparing' effect steming from reduced HGPRT activity (Graf et. al. 1976).

Other factors are also known to affect PRPP levels. When human lymphoblasts were deprived of glutamine, an essential precursor for purine biosynthesis (Mahler and Cordes 1971), intracellular PRPP levels fell dramatically (Skaper et. al. 1976). The authors suggested that this phenomenon may be the result of a lack of a substrate for PRPP synthetase. Though pyrimidines are not known to inhibit eucaryotic PRPP biosynthesis (Wood and Seegmiller 1973), purine biosynthesis in human fibroblast was inhibited by exogenous addition of orotic acid. The data suggest that this inhibitory effect of orotic acid is most likely related to a depletion of intracellular PRPP (Kelley et. al. 1970).

(ii) 5-Phosphoribosyl-1-Pyrophosphate Amidotransferase (EC 2.4.2.14)

The first enzymatic reaction in nearly all metabolic pathways is generally an important regulatory protein (Wyngaarden 1972) and PRPP amidotransferase, the first enzyme in the de novo purine biosynthetic pathway is no exception. This enzyme catalyses the synthesis of

phosphoribosylamine (PRA) by the following reaction:

$$\text{PRPP + Glutamine} \xrightarrow[Mg^{2+}]{H_2O} \text{Phosphoribosylamine + Glutamate + PPi}$$

$$\text{PPi} \longrightarrow 2\text{Pi}$$

Studies with both partially purified enzymes and intact mammalian cells suggest that the overall rate of de novo purine synthesis is dependent on the levels of PRA (Henderson 1972).

Two other enzyme activities capable of catalyzing the synthesis of PRA have also been detected. The first of these, known as PRPP aminotransferase, utilizes ammonia rather than glutamine as the substrate (Reem 1972). Whether this activity represents a distinct new protein or is a subunit of PRPP amidotransferase remains unknown. The physiological importance of the second enzyme activity, ribose-5-phosphate aminotransferase which has ribose-5-phosphate, ATP and ammonia as substrates for PRA synthesis has yet to be shown in vivo (Reem 1968).

The glutamine-dependent PRPP amidotransferase requires Mg^{2+} or Mn^{2+} as a cofactor and is stabilized by high Pi levels (Rowe and Wyngaarden 1968). This enzyme from avian livers has a molecular weight (MW) of about 210,000 and twelve atoms of non-haem iron (Caskey et. al 1964; Hartman 1963). The 210,000 MW component from pigeon liver in

turn consists of four identical subunits. It is dissociable into two 100,000 MW species by dilution of the enzyme preparation and into the 50,000 MW units by thiol reagent treatment. In the presence of AMP and GMP, the enzyme is converted into the inactive 100,000 MW species (Rowe and Wyngaarden 1968) while high concentrations of PRPP will reverse this process (Itoh et. al. 1976). In contrast, the PRPP amidotransferase from human placenta exists in two MW forms of 133,000 and 270,000 (Holmes et. al. 1973b). The enzyme activity correlates directly with the amount of it present in the 133,000 MW form, suggesting that this smaller enzyme species is the active form (Holmes et. al. 1973b). Thus purine ribonucleotides which inhibit the human PRPP amidotransferase converts it into the large MW form while PRPP reverses the process (Holmes et. al. 1973b). Though the human and avian PRPP amidotransferase show rather distinct structural and catalytic differences, both are equally sensitive to inhibition by the end-products of the purine biosynthetic pathway i.e. IMP, AMP and GMP. This phenomenon is very specific as purine 2' and 3'-ribonucleotides, 5'-deoxyribonucleotides, ribonucleosides, free bases and pyrimidine compounds were ineffective (Wyngaarden and Ashton 1959; Holmes et. al. 1973a).

(iii) Regulation of AMP and GMP Synthesis from IMP

IMP can be derived from the de novo purine biosynthetic pathway or through the salvage of hypoxanthine by HGPRT (Mahler and Cordes 1971). In vitro studies have revealed that the intracellular concentration of IMP is very low in mammalian tissues and maybe limiting for adenylosuccinate synthetase (EC 6.3.4.4.) and IMP dehydrogenase (EC 1.3.1.14) (van der Weyden and Kelley 1974; Holmes et. al. 1974). Using cultured human lymphoblasts, it was observed that the de novo IMP branch is coordinately regulated (Hershfield and Seegmiller 1976). For example if guanine is added exogenously to the growth medium, the synthesis of GMP is selectively inhibited while the other branch leading tp AMP synthesis is stimulated. Exogenously added adenine produced a similar but complementary effect. The effects of exogenous adenine and guanine on the utilization of de novo derived IMP for AMP and GMP synthesis can be explained by a simple selective feedback inhibition of one branch by an end product and the simultaneous stimulation of the other branch pathway owing to the increased availability of the 'competition-limited' substrate, IMP (Hershfield and Seegmiller 1976).

(iv) Induction, Repression and Derepression of De Novo Purine Biosynthetic Pathway Enzymes

Data on the induction, repression and derepression of the enzymes of the de novo purine biosynthetic pathway in mammalian cells remain very sketchy. McFall and Magasanik (1960) reported that enzymes of the entire purine biosynthetic pathway may be repressed in mouse L cells which have been cultured for several generations in the presence of adenine or guanosine. Apparently under such conditions all the purines of the intracellular soluble pools were derived exclusively from the exogenously supplied purine. In whole animal studies (Reem and Friend 1967), PRPP amidotransferase activity, which is normally absent in mouse spleen, appeared four days following infection with Friend leukemia virus. The rise in activity peaked after six to nine days followed by a gradual decline. The mechanism of induction of the PRPP amidotransferase activity by the virus is unknown.

(b) Regulation of De Novo Pyrimidine Biosynthesis in Mammalian Cells and Tissues

The regulation of de novo pyrimidine biosynthesis in mammalian cells is both complex, and at times ambiguous. The first reaction that is unique to the de novo pyrimidine biosythetic pathway is the synthesis of carbamyl aspartate; the reaction is catalysed by aspartate transcarbamylase (EC 2.1.3.2). Mammalian aspartate transcarbamylase, however, has always been copurified with two other enzymic activities, carbamyl phosphate synthetase (EC 2.7.2.9) and dihydroorotase (EC 3.5.2.3) which catalyse the syntheses of carbamyl phosphate and dihydroortic acid respectively (Mori and Tatibana 1975; Kempe et. al. 1976; Ito and Uchino 1972). A recent study has shown that all three enzymes from SV_{40} transformed hamster cells are covalently linked as a multifunctional high molecular weight protein (Coleman et. al. 1977).

Persuasive data suggest that carbamyl phosphate synthetase may be an important regulatory enzyme because it has been shown to catalyse a rate-limiting step for pyrimidine biosynthesis (Hager and Jones 1967a; Hager and Jones 1967b). This enzyme is allosterically feedback

inhibited by UTP which is an end-product of the pyrimidine pathway (Levine et. al. 1971). Aspartate transcarbamylase, on the other hand is insensitive to pyrimidine nucleotide feedback inhibition (Smith et. al. 1972) though pyrimidine nucleosides are potent inhibitors (Bresnick 1963). Of interest is the observation that purine deoxyribonucleosides and deoxyribonucleotides strongly inhibited aspartate transcarbamylase activity (Bresnick 1962), suggesting that purine derivatives may be directly involved in regulating de novo pyrimidine synthesis. The last enzyme of the multifunctional enzyme complex, dihydroorotase, appears to be regulated, in vitro, like aspartate transcarbamylase (Coleman et. al. 1977) with respect to its inhibition by pyrimidine and purine derivatives (Bresnick and Blatchford 1964). Whether the activities of carbamyl phosphate synthetase, aspartate transcarbamylase and dihydroorotase are coordinately regulated by end-products of de novo pyrimidine synthesis remains unclear (Bresnick and Blatchford 1964) though the biosynthesis of the three enzymes of this enzyme complex are coordinately regulated (Kempe et. al. 1976).

Of the other enzymes involved in mammalian de novo pyrimidine biosynthesis, orotate phospho-ribosyltransferase (EC 2.4.2.10) may be another key control point because firstly , this enzyme utilizes

PRPP, a common intermediate required for both purine and pyrimidine biosynthesis (Mahler and Cordes 1971) and secondly it appears to be rate-limiting in Ehrlich ascites cells (Shoaf and Jones 1973). Recent studies have shown that both adenine and adenosine are toxic to cultured mammalian cells (Green and Chan 1973; Ishii and Green 1973). This phenomenon may be the result of a block of pyrimidine biosynthesis at the level of orotate phosphoribosyltransferase since orotate was found to accumulate under these conditions (Ishii and Green 1973). This may be the direct result of a reduced availability of PRPP for OMP biosynthesis owing to competition for this metabolite by the adenine salvage pathway. Alternatively, the resulting excessive accumulation of adenine deoxynucleosides and deoxynucleotides would inhibit the aspartate transcarbamylase activity (Bresnick 1962). Hoogenraad and Lee (1974) reported that de novo pyrimidine synthesis in rat hepatoma cells grown in continuous culture was inhibited by the addition of 0.5 mM uridine to the growth medium. The activity of orotate phosphoribosyltransferase decreased while the other enzymes for de novo pyrimidine biosynthesis were unaffected (Hoogenraad and Lee 1974). Because uridine, UMP and UTP did not affect the in vitro activity of

orotate phosphoribosyltransferase, this translation and transcription dependent inhibition of this enzyme may be due to a depletion of PRPP by excessive salvage of uracil from the breakdown of uridine. The actual mechanism of inhibition however remains obscure (Hoogenraad and Lee 1974).

The last enzyme for de novo UMP biosynthesis is orotidine-5'-phosphate decarboxylase (EC 4.1.1.23). This enzyme which has been detected in numerous mammalian tissues and cells (Smith et. al. 1972) is subjected to end-product inhibition by UMP and to a lesser degree by other purine and pyrimidine nucleotides (Appel 1968). UMP plays a central role in pyrimidine metabolism, particularly in its conversion to the other two pyrimidines, cytosine and thymine (Mahler and Cordes 1971).

Like de novo purine biosynthesis, very little is known about the regulation of the synthesis of the enzymes of the de novo pyrimidine biosynthetic pathway in mammalain tissues and cells. Besides the known coordinated regulation of the synthesis of the multifunctional enzyme complex described previously (Kempe et. al. 1976), it is now known that reticulocytosis leads to a complete loss of dihydroorotate dehydrogenase in the mature erythrocytes (Smith et. al. 1972).

(5) Human Genetic Diseases and Defective Purine and Pyrimidine Metabolism

(a) Gout

Numerous human genetic diseases are directly related to defects in the anabolism and catabolism of purines and pyrimidines. The classical of these is gout which is characterized by the deposition of sodium urate crystals in various limb joints. This disease is associated with an overproduction of purines leading to a hyperuricaemic state (Wyngaarden and Kelley 1972). For example, some patients have been reported to possess mutant 5-phosphoribosyl-1-pyrophosphate (PRPP) synthetases with either an increase in specific activity (Becker et. al. 1973) or a lack of feed-back resistance (Zoret et. al. 1975). Others lack various purine salvage enzymes leading to an accumulation of PRPP (Rosenbloom et. al. 1968). The end result of these enzymic defects is an acceleration of de novo purine biosynthesis and an excessive production of uric acid.

(b) Lesch-Nyhan Syndrome

A fairly rare human disorder associated with severe neurological abnormalities is the Lesch-Nyhan syndrome (Lesch and Nyhan 1964). This X-linked disease is related to a total or nearly total deficiency of hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity in various tissues and is accompanied by an enhancement of de novo purine synthesis (Rosenbloom et. al. 1968; Seegmiller et. al. 1967). The importance of HGPRT in human cellular metabolism was better appreciated after Adams and Harkness (1976) reported that there is a significant increase in HGPRT activities in the normal human cerebral cortex and medulla after the age of two years. This seems to correlate well with the chronological observation that in Lesch-Nyhan syndrome patients, a rapid deterioration in the behavioural and neurological abnormalities occur after this age (Nyhan 1973). But the biochemical relationship(s) between HGPRT deficiency and the neurological symptoms remains obscure.

(c) Immunodeficiency Syndromes

Two other human genetic disorders known as severe combined immunodeficiency diseases are associated with nucleic acid metabolism. Giblett et. al. (1972) first reported absence of adenosine deaminase (ADA, EC 3.5.4.4), which is involved in the conversion of adenosine to inosine and ammonia, in two young patients with T-cell and B-cell dysfunction. Subsequent studies have established at least two dozen families known to be afflicted with this disease (Hirschhorn et. al. 1975; Meuwissen and Pollara 1974). Because adenosine is toxic to cultured lymphoid cells (Green and Chan 1973) and because patients with ADA syndrome have elevated levels of adenine, adenosine and adenosine nucleotides (Mills et. al. 1976), it was thought that these purines are probably responsible for the severe lymphocytopenia observed in these patients. Carson et. al. (1977) recently, however, reported the presence of high activities of ADA and adenosine kinase that are specific for deoxyadenosine, deoxyinosine and deoxyguanosine in the lymphocytes of newborn human tissues. Simmonds et. al. (1978) then showed that deoxyadenosine was far more toxic to mitogen-

stimulated lymphocytes than adenosine. Contrary to the study by Mills et. al. (1976), two research groups have now reported that there is a substantial elevation of deoxyadenosine nucleotides and a decrease in ATP levels in the erythrocytes of two patients with severe combined immunodeficiency associated with a lack of ADA activity (Cohen et. al. 1978; Coleman et. al. 1978). It is now thought (Cohen et. al. 1978; Coleman et. al. 1978) that severe combined immunodeficiency disease may be due to the inhibition of DNA synthesis and hence mitogenesis of precursor T- and B-cells through the inhibition of ribonucleotide reductase by elevated levels of deoxyadenosine triphosphate (Moore and Hurlbert 1966). Whether elevated levels of deoxyATP also persists in the lymphocytes of these immunodeficient patients remains to be elucidated. However the enormous difficulty in obtaining sufficient lymphocytes for such studies from these patients is a drawback. This disease, if detected very early in infancy, may ultimately be amenable to treatment.

The other immunodeficiency disease, with a concommitant absence of purine nucleoside phosphorylase (EC 2.4.2.1), was first reported in 1975 (Giblett et. al. 1975). The disease is characterized by an impairment in T-cell immune functions (Giblett et. al 1975). Purine

nucleoside phosphorylase, which catalyses the reversible conversion of guanosine, deoxyguanosine, inosine and deoxyinosine to their respective bases (Parks and Agarwal 1972), is generally believed to function in vivo in the direction of nucleoside breakdown. This is a reasonable assumption because with the exception of deoxyguanosine (Anderson 1973), there are no known mammalian nucleoside kinases capable of converting guanosine, inosine and deoxyinosine to their respective nucleotides (Milman et. al. 1976). The four substrates of purine nucleoside phosphorylase have been found to be elevated in the body fluids of patients which lack this enzyme (Cohen et. al. 1976). DeoxyGTP moreover, has recently been observed in the erythrocytes of purine nucleoside phosphorylase-deficient, immunodeficient children, but not in normal controls (Cohen et. al. 1978). Studies show that of the four substrates of purine nucleoside phosphorylase, deoxyguanosine is the most toxic to mouse T-cell lymphoma cell lines (Chan 1978; Gudas et. al. 1978). The cytotoxic effect of deoxyguanosine was dependent on it being phosphorylated to deoxyGTP by deoxycytidine kinase (Gudas et. al. 1978). Furthermore, the extractable pool of deoxyCTP was markedly depressed in the presence of deoxyguanosine and its cytotoxicity could be reversed

by the simultaneous addition of deoxycytidine and hypoxanthine to the growth medium (Chan 1978). Owing to these findings, it has been suggested (Chan 1978; Gudas et. al. 1978) that the T-cell dysfunction in purine nucleoside phosphorylase deficient patients may be the result of an excessive accumulation of deoxyGTP in the lymphoid tissues of these patients. This deoxynucleotide is a known inhibitor of cytidine diphosphate reduction to dCDP (Moore and Hurlbert 1966) and de novo purine biosynthesis (Chan 1978). This conclusion of deoxyGTP toxicity is supported further by the observation that lymphoid tissues have the highest deoxycytidine kinase activities in the body and the greatest ability to phosphorylate deoxyguanosine (Durham and Ives 1969; Carson et. al. 1977). The relevance of these findings of an in vitro animal lymphoma model must await similar studies with human tissues.

In contrast to the combined immunodeficiency disease associated with an absence of adenosine deaminase activity (Giblett et. al. 1972), several cases of dominantly transmitted hereditary hemolytic anemia with elevated levels of erythrocyte adenosine deaminase has now been reported (Valentine et. al. 1977). This disease is associated with decreased ATP levels in the red blood

cells presumably due to the result of the high adenosine deaminase activity (Valentine et. al. 1977). This hemolytic phenomenon may thus be directly related to the lack of ATP for maintaining cellular metabolism in the non-nucleated erythrocyte which are incapable of de novo purine biosynthesis (Valentine et. al. 1977).

(d) Oroticaciduria and Xanthinuria

Two other human hereditary disorders directly related to defective purine and pyrimidine metabolism are xanthinuria and oroticaciduria. Xanthinuria is characterized by a deficiency of xanthine oxidase activity. The resulting high levels of serum xanthine and hypoxanthine lead to urinary xanthine stones formation and occasional myopathy (Wyngaarden 1972).

Oroticaciduria is associated with an inability to convert orotic acid to UMP due to a lack of orotidine 5'-phosphate decarboxylase activity in all patients while most others also have a defective orotate phosphoribosyltransferase (Zellner et. al. 1976) thus suggesting two forms of the syndrome. Patients suffering from oroticaciduria are characterized by retarded growth and development, anaemia and excessive orotic acid

excretion (Smith et. al. 1972). Replacement therapy using either uridine or pyrimidine nucleotides has helped alleviate the pathological symptoms (Smith et. al. 1972).

The biochemical mechanisms of some of these human genetic diseases related to aberrant purine and pyrimidine metabolism remain obscure. A better understanding of the regulation of purine and pyrimidine biosynthesis and the development of more precise methods for quantitating nucleobases, nucleosides and nucleotides will be necessary to delineate the biology of such human diseases.

MATERIALS AND METHODS

MATERIALS

(1) Organisms

Mammalian Cell Lines

CHO WT, CHO GAT$^-$, mouse 3T3 fibroblasts and the CHO purine salvage mutants, YH 21 and YHD 13 (originally from Dr. L. Chasin, Columbia University) were kindly supplied by Dr. J.A. Wright, Dept. of Microbiology, U. of Manitoba. Mouse L5178Y lymphoblasts and SV40 transformed mouse 3T3 cells were kind gifts from Dr. W. Hryniuk (Manitoba Cancer Treatment and Research Foundation, Winnipeg) and Dr. R. Sheinen (Ontario Cancer Institute, Toronto) respectively.The Lesch-Nyhan (On Ser) and normal human (El San) skin fibroblasts were obtained from the American Type Culture Collection, Rockville, Md., USA. All the rest of the cell lines studied were purchased from Flow Laboratories, Rockville, Md., USA.

Fungal Cells

The fungal strain from which HS3 was purified was obtained originally from Dr, J.S. Lovett, Purdue University, and designated Achlya sp. (1969) (LéJohn and Stevenson 1970).

(2) Growth Media, Sera and Culture-wares

Alpha-minimal essential medium (∝-MEM) was obtained from Flow Laboratories, Rockville, Md., USA, while Fisher's medium was prepared according to the method of Fisher and Sartorelli (1964).

Mycoplasma tested and virus screened dialysed and undialysed fetal calf sera were purchased from Grand Island Biological Co., Grand Island, New York.

Culture plates and tubes were obtained from either Lux Scientific Corporation, Ca., USA or Falcon, Ca., USA.

(3) Radioisotopes

All labelled chemicals were obtained from Amersham. The specific activities are reported in the appropriate 'Methods' and 'Results' sections. ^{32}P-ortho-phosphate (carrier free; 8 mCi/ml) was obtained in dilute HCl (pH 2-3).

(4) Miscellaneous Materials

(a) All chemicals used were of analytical grade and were purchased from one of the following: Sigma

Chemical Co., Difco Laboratories, Calbiochem, J.T. Baker Chemical Co. and Fisher Scientific.

(b) Bacto-trypsin from Difco Laboratories, Detroit, Michigan.

(c) NCS Tissue Solubilizer from Amersham/Searle Corp., Illinois.

(d) PEI-cellulose plates from Brinkman Instruments (Canada) Ltd.

(e) Gelman (25 mm) glass fibre filters, type A-E from Gelman Instrument Co., Michigan.

METHODS

(1) Growth of Cell Lines

All cell lines, except L5178Y mouse lymphoblasts, were routinely cultured in Brockway bottles at 37°C in a humidified 5% CO_2 atmosphere incubator. Cells were subcultured on reaching confluency, using 0.05% trypsin in phosphate buffered saline to dislodge them from the glass surface. L5178Y mouse lymphoblasts, which do not attach to solid surfaces, were grown as stationary cultures in 100 ml serum bottles. Subculturing was carried out when the cell density approached 4-5 X 10^5 cells/ml. The incubation conditions were as described above. The split ratios for the permanent cell lines varied while a 1:4 ratio was rigorously followed for all primary cell lines. Primary cell lines were used between passage numbers 3-35 for all experiments.

The growth medium used for culturing cells was α- minimal essential medium (α-MEM) (Stanners et. al. 1971). The only exceptions being for CHO GAT^- and L5178Y cell lines in which α-MEM plus adenine/adenosine (0.1 mM) and thymidine (0.1 mM) and Fisher's medium (Fisher and

and Sartorelli, 1964) respectively were substituted. In all cases, the growth media were supplemented with 10% fetal calf serum and penicillin and streptomycin added at 60.6 mg/litre and 68.6 mg/litre respectively.

(2) Growth, ^{32}P-Orthophosphate Labelling and Formic Acid Extraction of Cells for Analysis of HS Polyphosphorylated Nucleotides During Various Growth Conditions

Cells were grown to near monolayer stage as described above. After trypsinization, the cells were recovered by low speed centrifugation, plated in 60 X 15 mm plastic tissue culture plates and allowed to grow for at least 24 hr before use. Permanent and primary cell lines were used at densities of between 0.5-1.0 X 10^6 and 0.2-0.5 X 10^6 cells/plate respectively for all experiments. For ^{32}P-labelling studies, the cells were first washed with 5 ml of the appropriate pre-warmed medium containing 10% fetal calf serum. Three millilitres of the 10% dialysed fetal calf serum (DFCS) containing incubation growth medium was next added to each culture plate together with 50 µl of ^{32}P-orthophosphate. The cells were then incubated for various times under normal growth conditions.

For pulse labelling studies, the incubation time was 30 min. The labelling was terminated by aspirating the incubation medium off, followed by a 5 ml wash with ice-cold 0.9% NaCl. Cells were then rapidly frozen for later use or extracted immediately for at least 30 min at $4^{o}C$ with 200 µl of 1 M formic acid/plate. For ^{32}P-labelling studies of L5178Y mouse lymphoblasts, the cells were cultured as described. Aliquots of the cell suspension (3-4 X 10^{5} cells/ml) were dispensed into 17 X 100 mm Falcon plastic tubes and the cells recovered by low speed centrifugation. They were washed once with an equal volume of the appropriate medium and resuspended in the same medium to give a final density of between 0.15-0.3 X 10^{6} cells/ml. The experiment was initiated with the addition of 50 µl of ^{32}P-orthophosphate to 17 X 100 mm Falcon plastic tubes containing 3 ml of the cell suspension. Incubation was carried out in a $37^{o}C$ shaking water-bath. Labelling was terminated by low speed pelleting of the cells and the supernatant aspirated. Formic acid extraction of cells was as described above.

(3) Chromatography of ^{32}P-Labelled Formic Acid Extract from Mammalian Cells

Ten microlitres of the formic acid extract was spotted on H_2O-washed polyethyleneimine (PEI) cellulose plates and developed one-dimensionally with 1.5 M KH_2PO_4

at pH 3.65 by ascending chromatography until the solvent front reached 15 cm from the origin. Two-dimensional chromatography of formic acid extracts on PEI-cellulose plates was carried out by developing with 3.3 M ammonium formate and 4.2% boric acid, pH 7.0 in the first dimension. After a 15 min wash in methanol, followed by drying, chromatography in the second dimension was with the phosphate solvent as defined above. In both dimensions, the solvent fronts were allowed to migrate to 15 cm from the origin.

After chromatography, the PEI-cellulose plates were exposed to Kodak RP-14 Royal X-Omat films for 24-48 hr before development. The appropriate labelled areas were identified, cut out and placed in 10 ml Bray's (1960) scintillation fluid. The radioactivity was determined with a Beckman LS-230 liquid scintillation spectrometer.

(4) DNA, RNA and Protein Analysis

Cells for all these experiments were cultured as described when they were labelled with ^{32}P-orthophosphate for isolation and analysis of HS polyphosphorylated nucleosides (see sections 1 and 2 of 'Methods').

(a) DNA Pulse Labelling Experiments

Before initiating radioisotope labelling, the cells were first washed with 5 ml of the appropriate pre-warmed medium. Three millilitres of fresh medium containing 10% dialysed fetal calf serum (DFCS) and 2 μCi/ml ^{3}H-TdR (methyl-^{3}H-TdR, 27 Ci/m mol) were then added followed by a 15 min incubation. The pulse was terminated by removing the radioactive medium by aspiration. The cells were washed once with 5 ml of ice-cold 0.9% NaCl and then 5 ml of ice-cold 10% trichloroacetic acid (TCA) was added. After at least 1 hr at 4^{o}C, the cells in TCA were removed from the plate and the entire suspension filtered through a glass fibre filter (Gelman) followed by three successive washes of 5 ml of ice-cold 5% TCA and dried with 10 ml of 70% ethanol. The dried filter was dissolved in 1 ml of NCS tissue solubiliser for 1 hr at 45^{o}C, and the radioactivity determined following addition of 9 ml of toluene-based scintillant.

(b) Protein Pulse Labelling Experiments

The procedure was exactly as described for DNA pulse labelling studies. Protein was labelled with ^{3}H(4,5)L-leucine (1 μCi/ml; 1 Ci/m mol).

(c) Continuous RNA Labelling Experiments

The washing procedure of cells prior to initiation of labelling and processing for radioactivity determination were exactly as described for DNA pulse labelling experiments.

^{3}H(5, 6) Uridine (1 µCi/ml, 45 Ci/m mol, 5 µM final concentration) was added to the cells at zero time. At specific intervals, the labelling was terminated and ^{3}H-uridine incorporation into total RNA was determined. To correct for the possible incorporation of ^{3}H-uridine into DNA, after its conversion to thymidine, the following procedure as described by Skold and Zetterberg (1969) was used. Duplicate samples were taken for each time point, one for analysis of total radioactivity incorporated, the other for actual incorporation into RNA. The latter sample was processed as follows. The cells were flooded with 10% TCA, then scraped from the plates and particulate matter collected by centrifugation. The pellet was suspended in 1.5 ml 1M NaOH for 16-18 hr at 37^{o}C, neutralized with HCl, followed by reprecipitation with ice-cold 5% TCA. The precipitate was finally collected on a glass fibre filter and radioactivity determined as described for DNA synthesis (4a). The amount of label in RNA was calculated as the difference in radioactivity between the two samples.

(5) Determination of ^{14}C-Adenine Incorporation into Nucleosides and Nucleotides

Experimental procedures were exactly as described for ^{32}P-orthophosphate labelling studies. $^{14}C(8)$-Adenine (1.1 µCi/ml; 60 mCi/m mol) was added followed by a 2 hr incubation. Thereafter the cells were extracted with formic acid, as described under section (2) of 'Methods' and 15 µl spotted on Whatman #1 chromatography paper. The chromatogram, with appropriate standard nucleotide markers, was developed overnight with saturated $(NH_4)_2SO_4$-0.1 M sodium acetate-isopropanol (75:19:2) by descending chromatography. After drying, the relevant chromatographic strips were cut into 1 X 3 cm portions and radioactivity determined by counting in Bray's scintillant. The positions of the standards were observed under UV-light.

(6) Determination of ATP Levels Using the Firefly Luciferin/Luciferase Method

Cells were cultured and washed as described under sections (1) & (2) of 'Methods'. Three millilitres of the appropriate medium was added followed by a 2 hr incubation

under normal growth conditions. The incubation was terminated by aspiration of the medium, then the cells were washed with 5 ml ice-cold 0.9% NaCl followed by immediate freezing in an ethanol-dry ice-bath. The procedures for the extraction and assay of ATP were essentially similar to those described by Grummt et. al. (1977). One millilitre of ice-cold 50% ethanol was added to each frozen culture. After 1 hr at $4^{o}C$, the cells were scraped off the plate with a rubber 'policeman'. Suspensions were used directly for ATP determinations. Each vial of firefly lantern powder (Worthington Biochem. Corp., New Jersey, USA) was reconstituted with 5 ml of H_2O and 100 µl was used per reaction. This was made fresh each time. Other constituents of the assay in a final volume of 2 ml were 40 µl of the ethanol-cell suspension extract, 10 mM $MgSO_4$ and 10 mM glycylglycine buffer pH 8.0. The ATP standard curve was constructed with the same volume of 50% ethanol in each sample. The light emission from each reaction vial was determined for 60 sec by an ATP photometer, model 2000 (JRB Inc., California) at 15 sec following the addition of the ethanol-cell suspension to the complete assay mix.

(7) Isolation of HS3 From Achlya and CHO WT Cells

The method of growing Achlya for cold osmotic-shock treatment has been described (Cameron and LéJohn 1978). The crude osmotic-shock fluid containing HS3 was concentrated under vacuum to between 0.1-0.2 ml. The entire sample was finely streaked onto PEI-cellulose plates and chromatographed one-dimensionally using 1.5 M KH_2PO_4 pH 3.65 solvent and a sample of ^{32}P-labelled formic acid extract from Achlya included as marker. The areas on the chromatogram corresponding to the ^{32}P-labelled HS3 sample were then cut out following extensive washing of the plates with methanol to reduce the inorganic phosphate contaminant. After drying, unlabelled HS3 was eluted from the cut areas overnight at 4^0C with 50 mM triethylamine bicarbonate (TEAB) buffer. The eluate was concentrated under vacuum and washed several times with H_2O to remove residual TEAB buffer. The concentrated sample was desalted by passing it through a Sephadex G-10 column and eluting it with H_2O. The fractions containing the first UV-absorbing peak were pooled and concentrated to dryness. The residue was dissolved in 1 ml of H_2O and the concentration of HS3 determined by its A_{260} extinction, assuming a molar extinction coefficient of 14.5×10^3 and a MW of 1800 for HS3. The sample was then reconcentrated

to dryness and resuspended in an appropriate volume of H_2O for use.

For the isolation of HS3 from CHO WT cells, 8-10 Brockway bottles of CHO cells, at 3 X 10^7 cells/bottle were used. Each bottle of cells was first washed with 30 ml of glutamine-free α-MEM + 10% DFCS, followed by a 4 hr incubation under normal growth conditions with the above medium. The starvation was terminated by pouring off the medium, washing the cells with 20 ml of ice-cold 0.9% NaCl and then frozen. The frozen cells were then extracted with 1 M formic acid for 30 min at 4°C. The isolation and quantitation of HS3 from this extract were exactly as described above for Achlya.

(8) Isolation of DNA-Dependent RNA Polymerases From CHO WT Cells

CHO WT cells were cultured in suspension culture with continuous stirring at 37°C and were used when the culture was at mid-log phase i.e. 3-5 X 10^5 cells/ml. For the isolation of DNA-dependent RNA polymerases, approximately 2-6 X 10^8 cells were harvested by low speed centrifugation and washed once with 0.9% NaCl. All manipulations beyond this step were carried out at 4°C.

The washed cells were resuspended in Buffer B (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM EDTA, 25% glycerol (v/v), 1 mM dithiothreitol, 1.7 mM phenylmethylsulfonyl fluoride (PMSF) and 20 mM $(NH_4)_2SO_4$ at a concentration of 1 X 10^8 cells/ml of buffer. The cell suspension was sonicated five times for 30 sec periods with 60 sec cooling intervals with an Insonator model 1000 sonicator (Ultrasonic Systems Inc., Farmingdale, New York) using a microtip set at a power level of 5. The cell debris was removed by centrifugation at 12,000 X g for 10 min. The supernatant was next subjected to high speed centrifugation in a Beckman ultracentrifuge using a Beckman 40 Ti rotor. Centrifugation was for 1 hr at 40,000 rpm. Protamine sulfate at 10 mg/ml in Buffer A (identical to Buffer B except for the omission of 1 mM DTT, 1.7 mM PMSF and 20 mM $(NH_4)_2SO_4$) was added slowly, with continuous stirring, to the high-speed centrifugation supernatant, to a final concentration of 1 mg/ml protamine sulfate. The preparation was stirred for an additional 15 min and the precipitate sedimented at 12,000 x g for 15 min. The supernatant fraction was discarded and the pellet resuspended in Buffer C (essentially Buffer B plus 120 mM $(NH_4)_2SO_4$). After centrifugation at 12,000 X g for 15 min, the supernatant was saved and the pellet

re-extracted with Buffer C as just described. Both supernatant fractions were then pooled together.

The protamine sulfate eluate was diluted 1:6 with Buffer A to give a final concentration of 20 mM $(NH_4)_2SO_4$. The diluted fraction was adsorbed to a 1.5 X 6 cm column of DEAE-Sephadex A-25 and the resin washed with 2-3 column volumes of Buffer B. The proteins were eluted with a linear gradient of 40 mM to 500 mM $(NH_4)_2SO_4$ (500 ml total gradient volume) in Buffer A containing 1 mM DTT and 1.7 mM PMSF. Fractions of 0.8 ml were collected and RNA polymerase activity of each fraction was assayed by measuring the incorporation of ^{3}H-UTP into TCA-precipitable material using a reaction mixture consisting of 70 mM Tris-HCl, pH 7.5, 25 mM 2-mercaptoethanol, 2 mM $MnCl_2$, 0.5 mM CTP, GTP and ATP, 4 µCi of ^{3}H-UTP (36.8 Ci/m mol), 5 µg of denatured calf thymus DNA and 15 µl of enzyme fraction in a final volume of 60 µl. The reaction mixture was incubated at 30°C for 30 min before 40 µl of the assay mixture was spotted on a piece of Whatman #1 filter paper. The dried filter paper was washed sequentially for 10 min periods with ice-cold 10% TCA, 5% TCA (twice), 95% ethanol and acetone. After complete drying, the radioactivity on the filter was determined in 10 ml of a toluene-based scintillant using a Beckman LS-230 liquid scintillation spectrometer.

(9) Cell Permeabilization and Assay for RNA Synthesis

Cells were cultured as described previously in section (1) of 'Methods'. For permeabilization of CHO WT cells, the procedure of Lewis et. al.(1978) was followed, with minor modifications. Cells were first trypsinised, pelleted by centrifugation and then washed once with warmed α-MEM plus 10% fetal calf serum. The cells were resuspended at a concentration of 2-3 X 10^6 cells/ml and incubated for 30 min at 22°C in a permeabilizing buffer consisting of 1% Tween-80, 0.25 M sucrose, 0.01 M Hepes buffer (pH 7.2) and 2 mM dithiothreitol. The permeabilized cells were centrifuged and resuspended in the permeabilizing buffer at a cell density of 3 X 10^6 cells/ml. Each assay for the incorporation of ^{3}H-UTP into RNA by permeabilized CHO WT cells consisted of 300 µl of the cell suspension and 200 µl of the RNA assay mix as described by Castellot et. al. (1978). Essentially the RNA assay mixture consisted of 35 mM Hepes (pH 7.4), 80 mM KCl, 4 mM $MgCl_2$, 7.5 mM potassium phosphate (pH 7.4), 0.75 mM $CaCl_2$, 50 mM sucrose, 0.5 mM $MnCl_2$, 4 mM ATP, 0.25 mM CTP and GTP and 0.01 mM UTP (2.5 µCi/sample). The cell-assay mix was incubated at 37°C in a shaking water-bath. The reaction was terminated by centrifuging the

cells, removing the supernatant by aspiration and 5 ml of ice-cold 5% TCA added to the pellet. After at least 1 hr at 4°C, the precipitate was filtered through a glass fibre filter followed by four successive washes of 5 ml ice-cold 5% TCA and three 5 ml washes using 70% ethanol before drying. The filter was processed for radioactivity determination as described for section (4a) of 'Methods'.

(10) Assay of RNA Synthesis By Permeabilized CHO WT Cells In the Presence of Achlya HS3

Preparation of permeabilized cells and the RNA assay procedure were as described previously. Achlya HS3, at a final concentration of 100 µg/ml was added to the appropriate samples. The incubation time for the assay was 1 hr at 37°C.

RESULTS

(1) HS3 Synthesis by CHO WT Cells

Studies were carried out to determine if cultured CHO WT cells can produce the dinucleoside polyphosphates , HS1, 2 and 3, which were previously found in various fungi (LeJohn et. al. 1975) and characterized recently by McNaughton et. al. (1978). HS compounds accumulated in significant quantities when fungal cells are starved of nutrients and especially so when glutamine is absent or being depleted in a growing medium (LeJohn et. al. 1978). Glutamine is present in most synthetic media at concentrations of between 2-4 mM; which is usually 5-10 fold greater than that of any of the other amino acids added as supplements (Stanners et. al. 1971; Fisher and Sartorelli 1964). It is an essential amino acid, for growth of cultured mammalian cells is rapidly arrested either upon its removal or upon its depletion (Eagle et. al. 1955; Ley and Tobey 1970; Pardee 1974). This is not unexpected considering the involvement of glutamine in numerous cellular processes (Stadtman 1973).

Since stringent bacterial strains produce ppGpp and pppGpp in response to deprivation of an essential amino acid (Cashel 1969; Cashel and Gallant 1969), it was decided to use a similar approach and

see if glutamine withdrawal would precipitate the accumulation of these HS compounds in mammalian cells.

As illustrated in fig. 3, panel IA, only HS3 was found in appreciable amounts in cells exposed to glutamine-free growth medium. In the presence of 2 mM glutamine, HS3 remained at low levels (fig. 3, panel IB). Two other very faintly ^{32}P-labelled spots appeared to chromatograph at regions where fungal HS1 and HS2 would normally be found. However, from the labelling patterns, HS1 and HS2 levels appeared to be unaffected by changes in the supply of glutamine. The nature of these two ^{32}P-labelled entities has not been investigated.

Figure 4 is an autoradiogram of a formic acid extract from ^{32}P-labelled glutamine-deprived CHO WT cells chromatographed two-dimensionally on PEI-cellulose plate. As can be observed, HS3 migrates subsequent to GTP in both dimensions. This two-dimensional chromatographic system was utilized occasionally to check for the purity of the presumptive HS3 obtained by the

Figure 3: Effect of L-glutamine deprivation on HS3 levels in CHO WT cells. The growth of cells was as described in 'Methods'. The cells were washed once with 5 ml of prewarmed α-MEM supplemented with or without glutamine plus 10% DFCS. Three millilitres of the appropriate medium containing 50 µl of ^{32}Pi was then added to each plate, followed by a 2 hr incubation. The labelling was terminated by removal of the medium by aspiration and washing of the cells with 5 ml of ice-cold 0.9% NaCl. The cells were then extracted for 30 min at $4^{o}C$ by the addition of 0.2 ml of 1 M formic acid to each plate. For chromatography, 10 µl of the formic acid extract was applied on PEI-cellulose thin layer plates and developed by ascending chromatography. The procedures for chromatography and the determination of the levels of ^{32}P-labelled compounds have been described in the 'Methods'. (IA) - glutamine; (IB) + glutamine

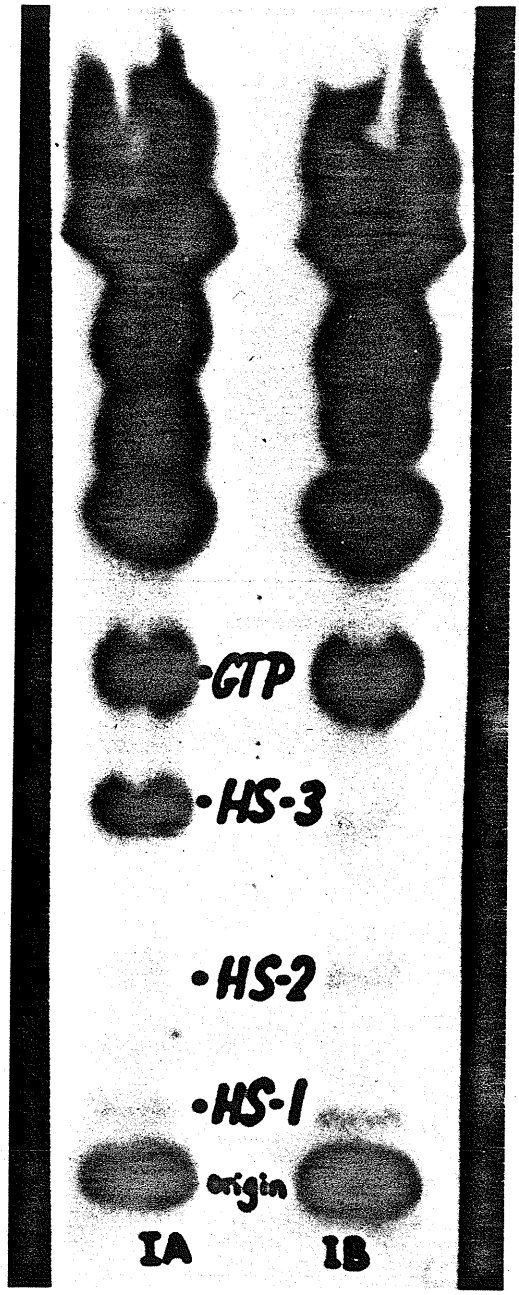
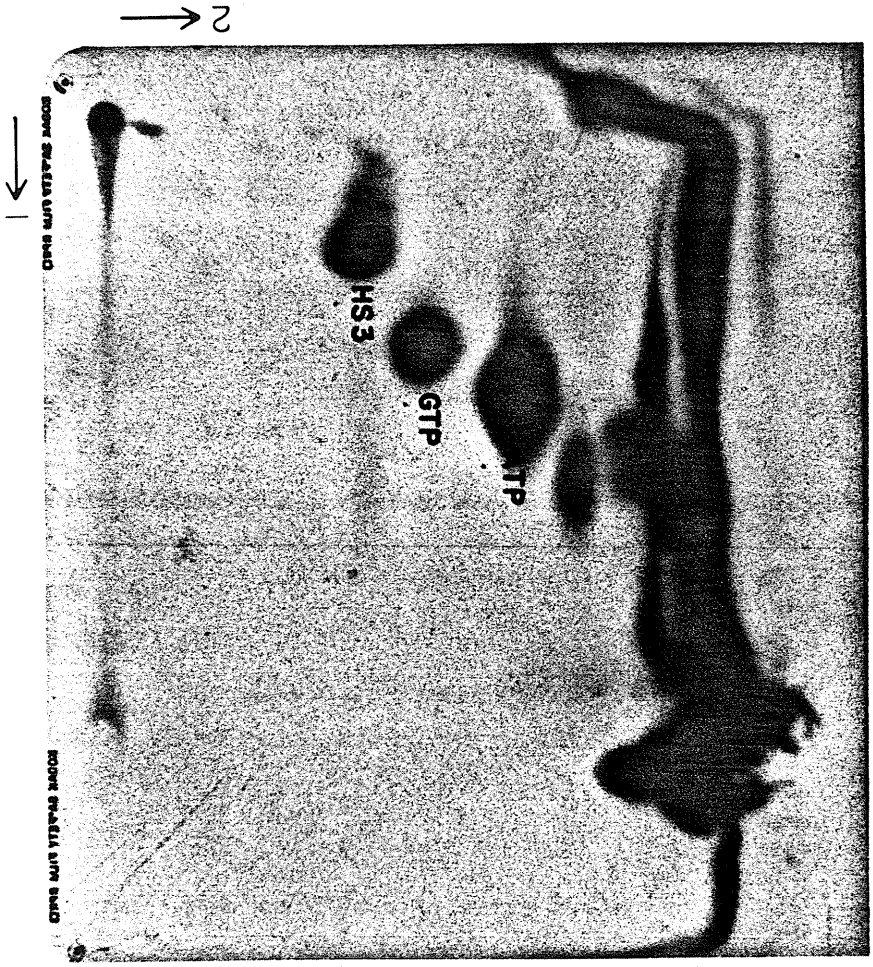

GTP
HS-3
HS-2
HS-1
origin
IA
IB

Figure 4: Autoradiogram of a two-dimensional chromatogram of 1 M formic acid extract of CHO WT cells starved for glutamine for 2 hr. ^{32}Pi labelling procedure was as described in the legend for fig. 3. The first dimension ($\longrightarrow$ 1) was developed in 3.3 M ammonium formate and 4.2% boric acid at pH 7.0. After chromatography in the first dimension, the plate was washed in methanol for 15 min and then dried. The second dimension ($\longrightarrow$ 2) was developed in 1.5 M KH_2PO_4, pH 3.65. The solvent front was allowed to migrate about 15 cm from the origin in both dimensions.


2
1
HS3
GTP
ITP

one-dimensional 1.5 M KH_2PO_4 solvent system. In all instances, the ^{32}P-labelled HS3 obtained one-dimensionally was totally recoverable by the two-dimensional chromatographic system. This, together with physical and chemical analyses of mammalian HS3 isolated from PEI-cellulose plates suggest that the ^{32}P-labelled entity with a R_f value of 0.36 (table 1) consists essentially of HS3.

Mammalian HS3 has been found to co-chromatograph with fungal HS3 as shown by their R_f values in table 1. Also, chemical and physical analyses of mammalian and fungal HS3 suggest that they are similar, if not identical molecules (table 2). Bacterial ppGpp and pppGpp did not co-chromatograph with mammalian and fungal HS nucleotides either one- or two-dimensionally (table 1). At no time in these studies were ppGpp and pppGpp ever detected in mammalian cells cultured under different growth conditions.

TABLE 1

R_f Values of Nucleotides on PEI-Cellulose Plates.

Nucleotide	1st Dimension	2nd Dimension
HS3 (Achlya)	0.22	0.35
HS3 (Mammalian)	0.21	0.36
ppGpp (MS I)	0.33	0.28
pppGpp (MS II)	0.29	0.17
GTP	0.38	0.45

1st dimension developed by ascending chromatography with 3.3M ammonium formate and 4.2% boric acid, pH 7.0.
2nd dimension developed by ascending chromatography with 1.5M KH_2PO_4, pH 3.65.

TABLE 2

Comparison of the properties of Achlya and mammalian HS3 (modified from Lewis et. al. 1977).

Property	Achlya HS3	Mammalian HS3 (CHO WT Cells)
R_f[1]	0.22[a](0.35)[b]	0.21(0.36)
DEAE-Sephadex-A25 elution at :		
(i) pH 8.0	1.1M	1.1M
(ii) pH 3.6	0.28M	0.27M
λmax at pH 7.0	260nm	260nm
λmin at pH 7.0	232nm	231nm
$A_{250/260}$ at pH 7.0	0.84	0.84
$A_{280/260}$ at pH 7.0	0.45	0.41
$A_{290/260}$ at pH 7.0	0.16	0.16
Resistance to alkaline phosphatase*	resistant	resistant
Combined treatment with phosphatase and pyrophosphatase	degraded	degraded
Components		
adenosine	+	+[2]
uridine	+	+[2]
glutamate	+	+
8 phosphates	+	+
mannitol	+	N.T.
sugar X	+	N.T.

[1] Two dimensional chromatography on PEI-cellulose as descibed in 'Methods'.

[a] 1st dimension. [b] 2nd dimension.

[2] Also detected in HS3 isolated from Lesch-Nyhan human fibroblasts.

*Incubated at 37°C for 1 hr with bacterial alkaline phosphatase.

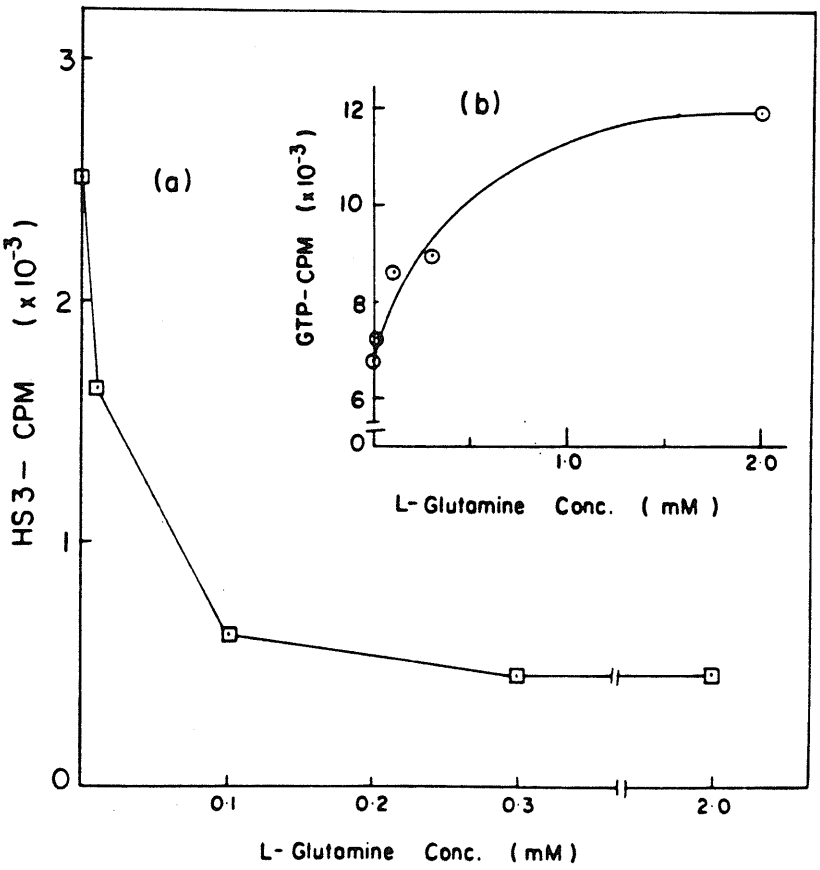
+Present, N.T. - not tested.

(2) Effect of Varying Concentrations of L-Glutamine on HS3 and GTP Synthesis by CHO WT Cells

As previously illustrated (fig. 3), the lack of 2 mM glutamine in the culture medium resulted in an accumulation of intracellular HS3 by CHO WT cells. An experiment was performed to determine the concentration effect of glutamine availability on HS3 and GTP levels in CHO WT cells. The results are illustrated in figures 5a and 5b. In a 2 hr labelling experiment, glutamine at 0.1 mM was adequate to reduce cellular HS3 to the level observed when a normal quantity (2 mM) of glutamine was supplied. When GTP levels were monitored, an inverse relationship between ^{32}P-labelled HS3 and GTP levels was observed (see figs. 5a and 5b), for an increase in HS3 led to a corresponding decrease in GTP and vice versa.

It can be observed from figures 5a and 5b that the ratios of ^{32}P-labelled $\frac{HS3}{GTP}$ (cpm) at glutamine concentrations of 0 μM and 2 mM were 0.37 and 0.047 respectively. However, fluctuations in the $\frac{HS3}{GTP}$ ratios especially for glutamine-starved CHO WT cells have been observed. The average $\frac{HS3}{GTP}$ ratio was 0.65. These variations in the $\frac{HS3}{GTP}$ ratios were similar to those of other cell lines that were studied.

Figure 5: Effect of varying glutamine concentrations (0-2 mM) on ^{32}Pi incorporation into (a) HS3 and (b) GTP in CHO WT cells. Experimental conditions were as described in the legend to fig. 3.


(a)
(b)
HS3- CPM (x10⁻³)
GTP-CPM (x10⁻³)
L- Glutamine Conc. (mM)
L- Glutamine Conc. (mM)
0
1
2
3
6
8
10
12
0·1
0·2
0·3
1·0
2·0

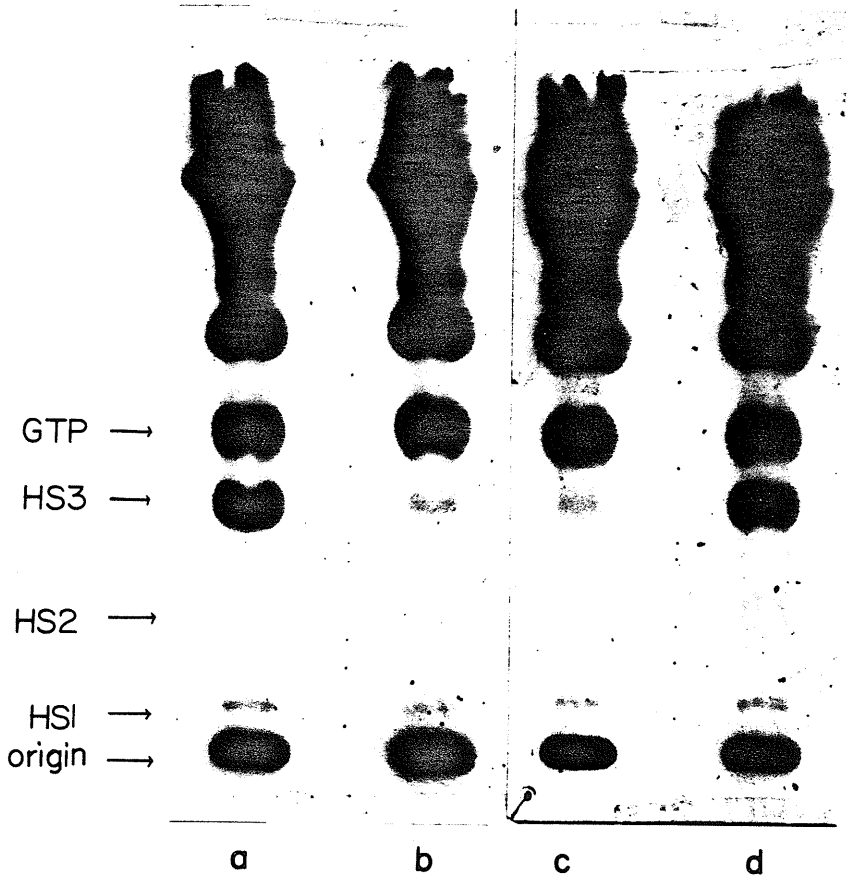
(3) Effect of L-Glutamine or L-Isoleucine Deprivation on HS3 Metabolism by CHO WT Cells

To ascertain whether HS3 is produced in response to either a general deficiency in amino acids or specifically for glutamine (see fig. 3), CHO WT cells were deprived of another essential amino acid, isoleucine. This nutrient has been implicated in regulating the cell cycle of CHO cells (Ley and Tobey 1970). The results of fig. 6 show that isoleucine deprivation did not lead to an accumulation of intracellular HS3 (see panel b).

(4) Effect of L-Glutamine, Adenosine and Thymidine Deprivation on HS3 Metabolism by CHO GAT^- Cells

A CHO mutant which is auxotrophic for glycine, adenosine and thymidine (GAT^-) owing to defective folate metabolism (McBurney and Whitmore 1974) was utilized to further define HS3 metabolism. Since HS3 accumulation appeared to be triggered specifically by glutamine deficiency (see fig 6) and owing to the importance of this amino acid in cellular metabolism, particularly in its role in purine metabolism (Stadtman 1973; Mahler

Figure 6: Effects of glutamine (gln) and isoleucine (ile) starvation on HS3 accumulation by CHO WT cells. The autoradiogram shows the relative HS3 level in cells incubated under different growth conditions (a) -gln, +ile; (b) +gln, -ile; (c) +gln, +ile; (d) -gln, -ile. The experimental procedures were as described in the legend to fig. 3.


GTP
HS3
HS2
HSl
origin
a
b
c
d

and Cordes 1971), it was reasoned that HS3 accumulation may be related to a lack of purines. An experiment was devised in which HS3 accumulation by the CHO GAT$^-$ mutant was monitored with respect to the availability of glutamine, adenosine and thymidine. The results show (table 3) that only the withdrawal of adenosine from the complete growth medium i.e. α-MEM plus 10% DFCS, adenosine and thymidine, resulted in a significant (four fold) accumulation of HS3 by this auxotroph.

(5) Effect of L-Glutamine Withdrawal and The Replenishment with Purines on HS3 Metabolism by CHO WT and CHO Purine Salvage Mutant Cells

The preceding results (table 3) suggested that HS3 accumulation in cultured mammalian cells may be induced by a lack of an intracellular supply of purines. It can be hypothesized that cells with blocked de novo purine biosynthesis and lacking purine salvage enzymes would exhibit elevated HS3 levels even when supplied with an exogenous purine. 'Wildtype' CHO and CHO purine salvage mutants were therefore examined for their ability to accumulate HS3 during glutamine starvation , when

TABLE 3

Effect of L-glutamine, adenosine and thymidine availability on HS3 metabolism by CHO GAT$^-$ cells.

	HS3(expressed as % of control)
+ glutamine + adenosine + thymidine	100
- glutamine + adenosine + thymidine	96
+ glutamine + adenosine - thymidine	96
+ glutamine - adenosine + thymidine	437
+ glutamine - adenosine - thymidine	413

The control is the amount of ^{32}P-orthophosphate incorporated into HS3 after a 2 hr incubation in complete α-MEM + 10% DFCS + 0.1 mM adenosine + 0.1 mM thymidine. The glutamine concentration used was 2 mM. Levels of HS3 were detected as described in the legend to fig. 3.

provided with an exogenous source of purines. Table 4 summarises the results obtained. Hypoxanthine, adenine and adenosine were effective in antagonising glutamine-less induction of HS3 accumulation in wildtype CHO cells. Both $HGPRT^-$ salvage mutants, YH 21 and YHD 13 however, were unable to do likewise when supplied with hypoxanthine; up to 2 mM hypoxanthine was without effect. By contrast, adenine and adenosine prevented HS3 from accumulating in both types of purine salvage mutants during glutamine deprivation. It was surprising that YHD 13, which is $HGPRT^-/APRT^-$, was able to utilize adenine and adenosine to depress HS3 levels. Under normal growth conditions, the purines mentioned had negligible effects on the levels of HS3 in all three cell lines (see table 4).

TABLE 4

Effect of L-glutamine and exogenous purines on intracellular HS3 levels in CHO WT and CHO purine salvage mutants.

Cell line	HS3 as % of control
'Wild type' CHO	10.9 (+ L-gln)
	9.7 (+ L-gln + 0.36 mM hypoxanthine)
	7.4 (- L-gln + 0.36 mM ")
	7.1 (- L-gln + 0.18 mM ")
	7.9 (- L-gln + 0.3 mM adenosine)
	7.9 (+ L-gln + 0.3 mM ")
	10.5 (- L-gln + 0.3 mM adenine)
	11.3 (+ L-gln + 0.3 mM ")
YH 21 ($HGPRT^-$)	104.9 (- L-gln + 0.3 mM hypoxanthine)
	11.5 (- L-gln + 0.3 mM adenine)
	12.0 (+ L-gln + 0.3 mM ")
YHD 13 ($HGPRT^-$ /$APRT^-$)	104.7 (- L-gln + 0.3 mM hypoxanthine)
	103.3 (- L-gln + 1.0 mM ")
	101.8 (- L-gln + 2.0 mM ")
	8.0 (- L-gln + 0.3 mM adenine)
	9.3 (+ L-gln + 0.3 mM adenosine)

The control for each cell type is the amount of ^{32}Pi incorporated into HS3 after a 2 hr incubation in L-glutamine (L-gln) -deficient medium and is expressed as 100%. Hypoxanthine, adenine and adenosine were added to the growth medium (± L-glutamine) at the start of all the experiments. YH 21 is a hypoxanthine/guanine phosphoribosyltransferase CHO mutant and YHD 13 is a hypoxanthine/guanine phosphoribosyltransferase-adenine phosphoribosyltransferase CHO mutant. The experimental procedures were as described in the legend to fig. 3.

(6) 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

It was of interest to determine the influence of various purines and pyrimidines and antagonists of de novo purine biosynthesis on HS3 metabolism since it has become apparent that the physiological levels of this polyphosphorylated compound are somehow related to changes in the supply of purines.

Table 5 summarises the effects of various purine and pyrimidine compounds, methotrexate(MTX) and azaserine on the accumulation and depletion of HS3 in CHO WT cells and the two purine salvage mutants YH 21 and YHD 13. In the presence of glutamine (see under '+' columns) none of the exogenously added purines and pyrimidines altered significantly HS3 levels in these cell lines although hypoxanthine, adenine and adenosine slightly depressed the levels of this polyphosphorylated compound in CHO WT cells. In contrast both azaserine and MTX, which are potent inhibitors of de novo purine biosynthesis (French et. al. 1963; Blakley 1969), were active in elevating intracellular HS3 levels in both CHO WT and YHD 13 cells.

In the absence of exogenous glutamine, all the purine bases and nucleosides tested were very potent in reducing the quantity of HS3 accumulated by CHO WT cells (see under '-' columns); but in the case of YH 21 and YHD 13 cell lines hypoxanthine, inosine and guanosine were rather ineffective though adenine exhibited the same potency in these two cell lines as was for CHO WT cells. In the case of pyrimidines, thymidine, thymine and orotic acid did not antagonise the accumulation of HS3 while 5-fluorouracil [Its metabolic derivatives are known to inhibit thymidylate synthetase and other cellular functions (Heidelberger 1975)] , uridine and cytidine, in decreasing order of effectiveness, did. In comparison, allopurinol, a xanthine analogue which potently inhibits xanthine oxidase (EC 1.2.3.2) (Pomales et. al. 1963), was as effective as cytidine.

(7) Determination of ATP Levels in CHO WT Cells After Exposure to Various Growth Conditions and Metabolic Inhibitors

Results have been presented in the preceding sections which showed a correlation between an elevation

TABLE 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.

	Relative amounts of HS3 in cells		
	CHO WT	YH 21	YHD 13
Compound added[1]	(+ : -)glutamine[2]	(+ : -)glutamine	(+ : -)glutamine
nil	(1.00 : 1.00)	(1.00 : 1.00)	(1.00 : 1.00)
hypoxanthine	(0.70 : 0.09)	(0.90 : 1.10)	(1.02 : 1.10)
inosine	(1.03 : 0.10)	(1.00 : 0.90)	(1.05 : 0.90)
adenine	(0.75 : 0.13)	(1.20 : 0.12)	(1.00 : 0.09)
adenosine	(0.67 : 0.08)		
deoxyadenosine	(1.04 : 0.15)		
guanosine	(1.00 : 0.13)		(1.20 : 1.20)
cytidine	(1.10 : 0.55)		
thymidine	(1.10 : 1.21)	(1.10 : 1.20)	(1.10 : 1.10)
thymine	(0.96 : 1.01)		
uridine	(0.92 : 0.35)		(1.00 : 0.40)
5-fluorouracil	(0.90 : 0.11)		(0.77 : 0.17)
orotic acid	(1.10 : 1.03)		
azaserine	(1.70 : 1.33)		
methotrexate	(2.32 : -)		(1.80 : -)
allopurinol	(- : 0.56)		

The experimental procedures were as described in the legend to fig. 3.

[1] Compounds added to the medium at the start of all experiments, were used at 0.3 mM (final concentration) except for MTX (1 uM), allopurinol (100 ug/ml), and azaserine (50 ug/ml).

[2] $$+ = \frac{\text{(cpm:HS3 in cells + glutamine + compound)}}{\text{(cpm:HS3 in cells + glutamine)}} ;$$

$$- = \frac{\text{(cpm:HS3 in cells - glutamine + compound)}}{\text{(cpm:HS3 in cells - glutamine)}} .$$

cpm = counts/minute

of intracellular HS3 and a block in de novo purine biosynthesis elicited by either glutamine deprivation (fig. 3) or antimetabolite treatment (table 5). Further, there is an inverse relationship between HS3 and GTP levels (figs. 5a and 5b). Experiments were therefore performed to determine the fate of cellular ATP concentrations in cells exposed to conditions which resulted in an accumulation of HS3.

Glutamine deprivation and the addition of either MTX or azaserine led to moderate decreases in ethanol-extractable ATP as determined by the firefly luciferin/luciferase assay (table 6). MTX, at 10 μM, lowered intracellular ATP level by about 12% while glutamine starvation and azaserine treatment depressed ATP concentrations by 20% and 30% respectively.

(8) Effects of Glutamine Deprivation or Sufficiency on ^{14}C-Adenine Incorporation Into Adenine Nucleoside and Nucleotides by CHO YHD 13 Cells

Since glutamine deprivation resulted in a decrease in both cellular ATP (table 6) and GTP (fig. 5b), a 2 hr labelling study was carried out to determine the relative incorporation of ^{14}C-adenine into adenine

TABLE 6

ATP concentrations, as determined by the firefly luciferin/luciferase assay method, in CHO WT cells after exposure to different growth conditions and metabolic inhibitors.

Growth Condition	ATP (% of control)
Complete growth medium	100
Complete growth medium - glutamine	79.9
Complete growth medium + 10 μM MTX	88.2
Complete growth medium + 50 μg/ml azaserine	69.9

The control, which is expressed as 100%, is the ATP level in cells incubated in complete growth medium i.e. α-MEM + 10% DFCS. The ATP concentrations were determined 2 hr after exposure to specific growth conditions and metabolic inhibitors.

nucleoside and nucleotides by glutamine-starved and unstarved cells. Figure 7 represents a one-dimensional paper chromatograph showing the levels of acid-extractable ^{14}C-adenine labelled nucleoside and nucleotides from YHD 13 cells. Glutamine-starved cells had the label accumulating predominantly in adenosine, AMP and ADP with hardly any in ATP. When glutamine was supplied, the label was incorporated almost entirely in adenosine and ATP which contained the bulk of the label supplied.

(9) Effects of L-Glutamine and Adenosine Deprivation and Replenishment on Rate of Accumulation and Pool Size of HS3

Since it was demonstrated that glutamine and adenosine withdrawal caused the accumulation of HS3 in CHO WT and CHO GAT$^-$ cells respectively (fig 3; table 3), experiments were performed to determine the changes in the rate of accumulation and pool size of this molecule when such cells were subjected to these same nutritional conditions. Figure 8 illustrates the results of an experiment in which ^{32}Pi was used to pulse label CHO WT cells during

glutamine starvation and the rate of labelling HS3 determined. The rate of HS3 accumulation increased by 5-6 fold in 30 min over the control culture and was subsequently maintained at this rate for at least 5 hr. From 6 hr onwards, the rate of HS3 accumulation declined slowly reaching control values by 30 hr.

In a continuous ^{32}P-labelling experiment, as shown in fig 9a, the intracellular pool of HS3 accumulated by CHO WT cells increased rapidly upon initiation of starvation. HS3 level increased five to six fold above the control culture by 5-7 hr and this was followed by a slow decline to control level. In a similar experiment (fig. 9b), HS3 accumulated by CHO GAT^- cells during adenosine starvation followed essentially the same pattern as that observed for CHO WT cells during glutamine deprivation.

When CHO WT cells that had been starved for 2 mM glutamine were refed with this amino acid, the high intracellular HS3 level decreased rapidly to control levels within 15 min. This is shown in fig. 10a. A similar phenomenon was observed (fig. 10b) when adenosine-starved CHO GAT^- cells were replenished with 0.1 mM of this nucleoside.

Figure 7: Chromatographic profiles of one-dimensional chromatograms of 1 M formic acid extracts obtained from YHD 13 cells incubated in growth medium with (⊙——⊙) and without (⊡——⊡) 2 mM glutamine and labelled with 3.3 µCi of ^{14}C-adenine [(8-^{14}C)adenine; 60 mCi/m mol] per plate for 2 hr. The experimental procedures for growth, labelling and formic acid extraction of cells were as described in the legend to fig. 3. The chromatography method has been described in the 'Methods'.

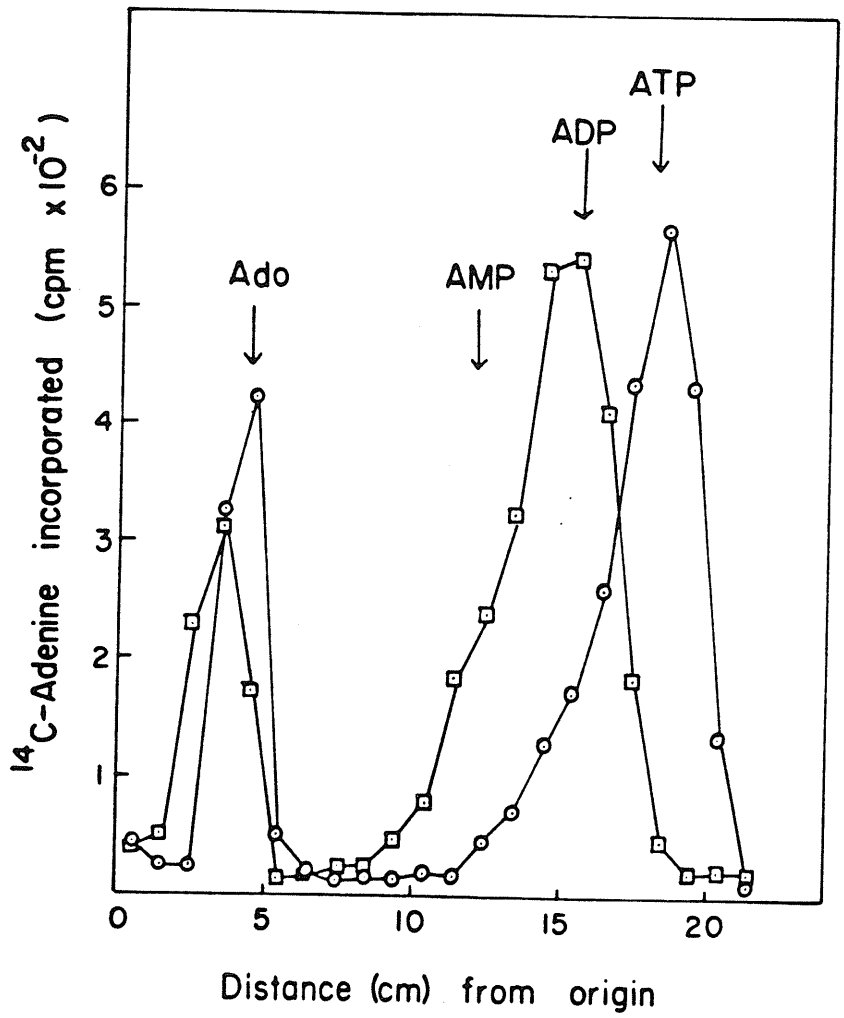

ATP
ADP
Ado
AMP
^{14}C-Adenine incorporated (cpm x 10^{-2})
1
2
3
4
5
6
0
5
10
15
20
Distance (cm) from origin

Figure 8: Effect of glutamine starvation on the rate of HS3 accumulation in CHO WT cells. The experimental protocol was as described in legend to fig. 3 except that the cells were pulsed with ^{32}Pi for 30 min at the intervals specified. HS3 : (⊙——⊙), minus 2 mM glutamine; (⊡——⊡), plus 2 mM glutamine.

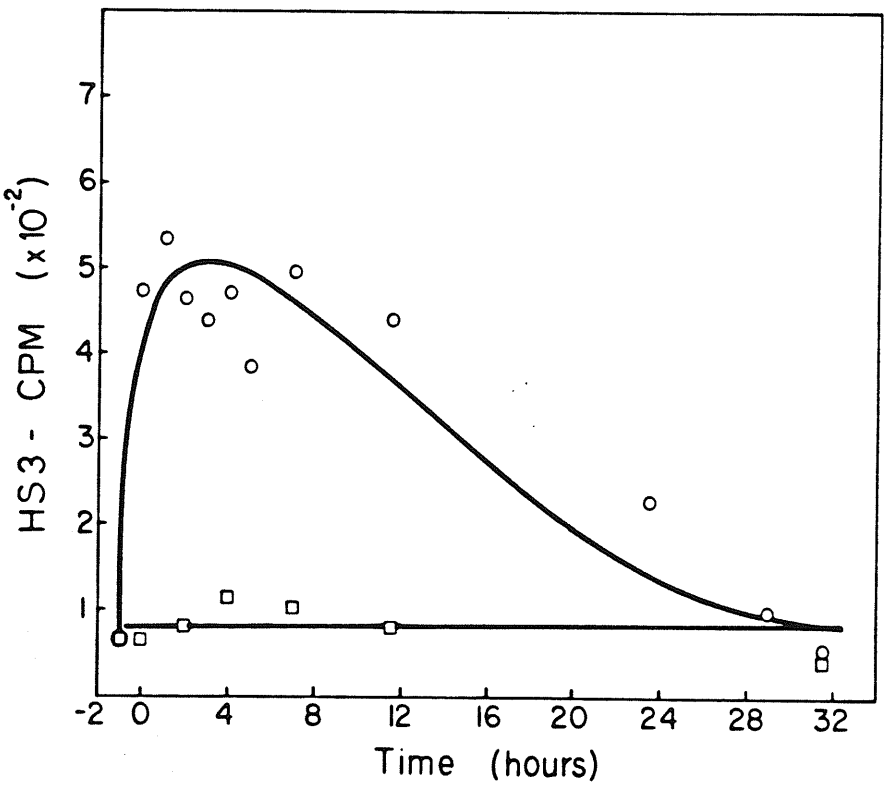

HS3 - CPM (x10^{-2})
7
6
5
4
3
2
1
-2
0
4
8
12
16
20
24
28
32
Time (hours)

Figure 9: Effect of nutrient (glutamine or adenosine) starvation on intracellular HS3 pool sizes in CHO cell lines. The experimental protocol was as described in legend to fig 3. In these continuous labelling experiments, ^{32}Pi was added to the cells at zero time and samples taken at the times specified. (a) CHO WT cells: HS3: (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine. (b) CHO GAT$^-$ cells: HS3: (⊙——⊙), minus adenosine; (⊡——⊡), plus adenosine.

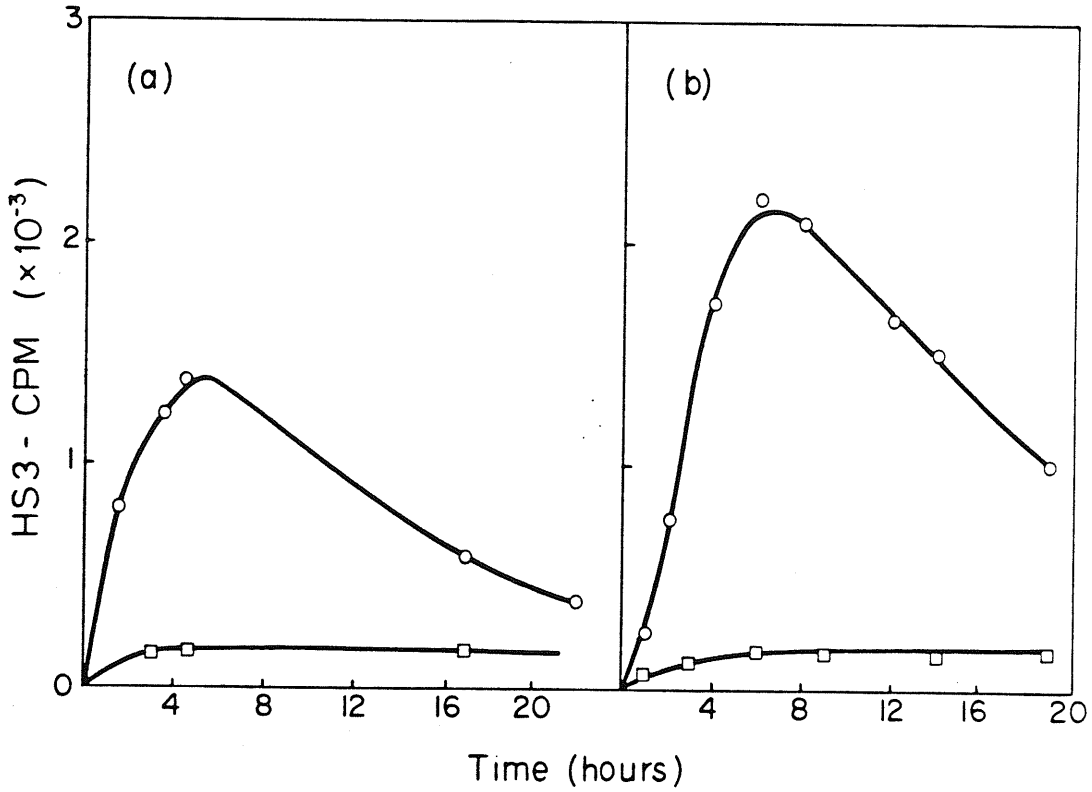
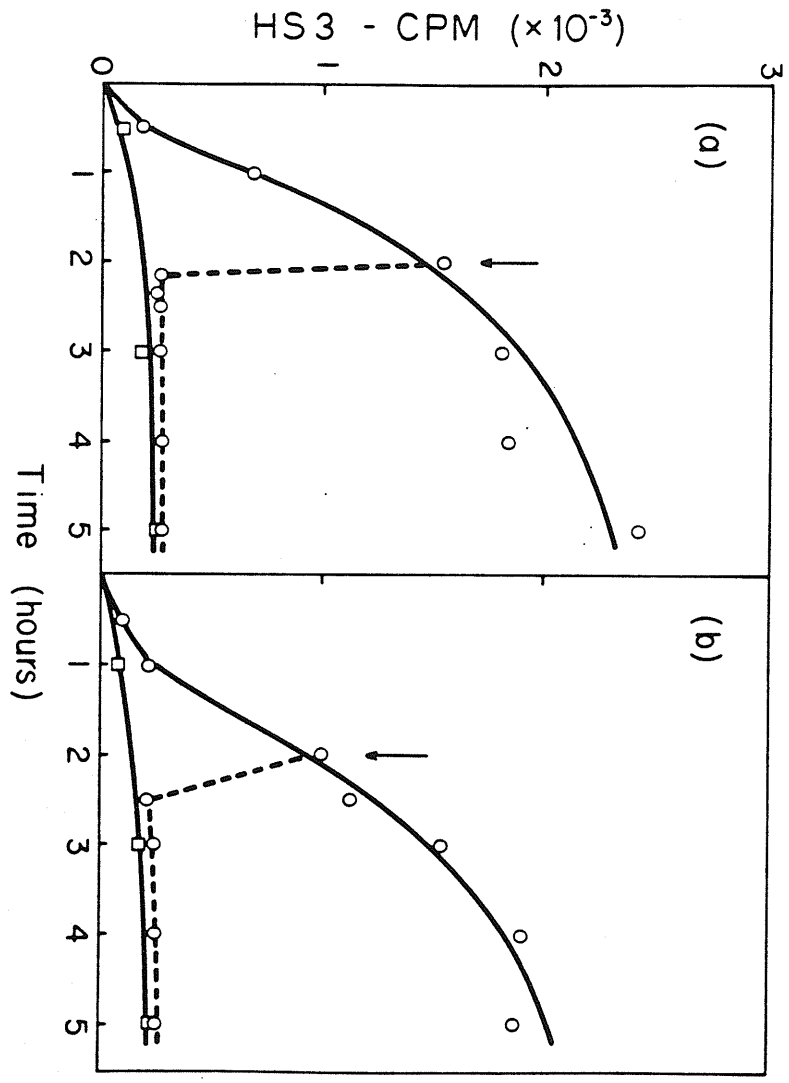

(a)
(b)
HS3 - CPM (×10⁻³)
0
1
2
3
4
8
12
16
20
Time (hours)

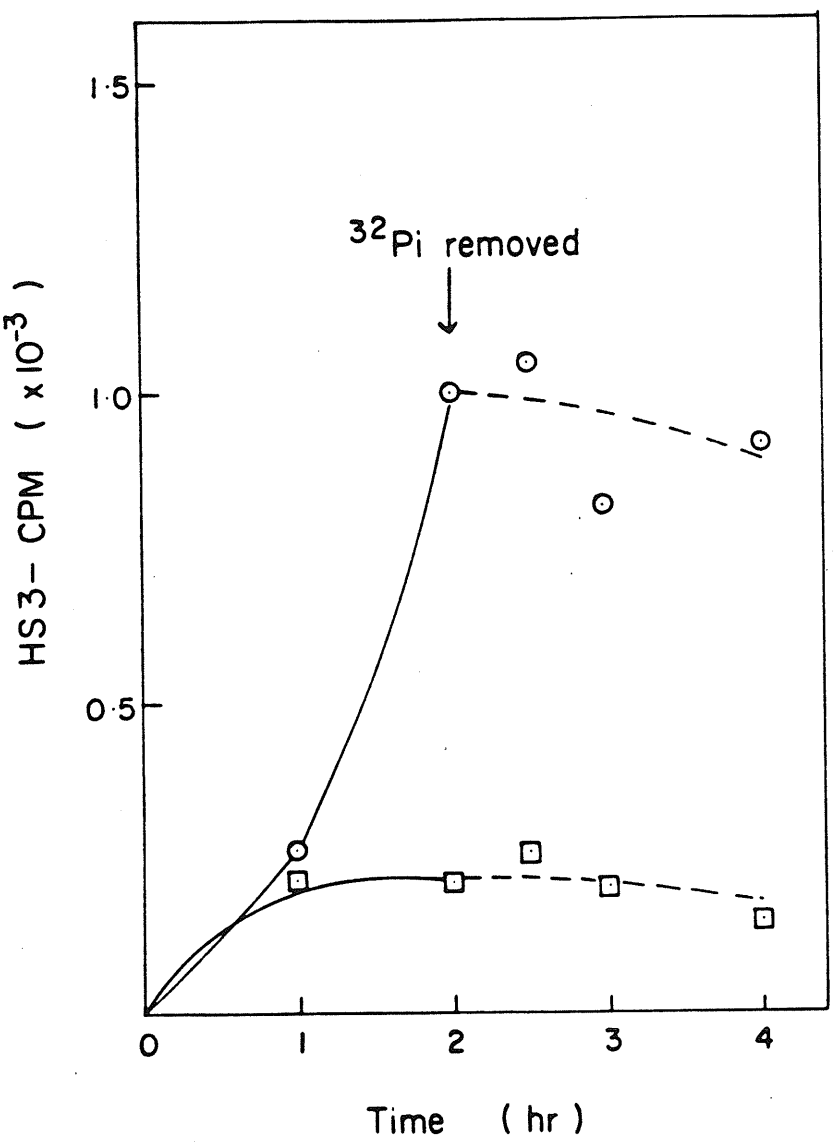
Figure 10: Replenishment of limiting nutrient (glutamine or adenosine) to CHO cell lines and its effect on HS3 pools. Experimental procedure as described in legend to fig. 3. (a) CHO WT cells: HS3: (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine; (⊙- - - -⊙), glutamine (2 mM) added to glutamine-starved cultures at the time indicated by the arrow. (b) CHO GAT^- cells: HS3: (⊙——⊙), minus adenosine; (⊡——⊡), plus adenosine; (⊙- - - -⊙), adenosine (0.1 mM) added to adenosine-starved cells at the time indicated by the arrow.


HS 3 - CPM (×10⁻³)
0
1
2
3
(a)
(b)
Time (hours)
1
2
3
4
5

(10) Fate of ^{32}P-Labelled HS3 Accumulated by CHO WT Cells During Glutamine Deprivation Following ^{32}Pi Withdrawal From the Incubation Medium

As illustrated by ^{32}P-labelling studies (figs. 8 and 9a), glutamine starvation of CHO WT cells resulted in an increase in the rate of accumulation and pool size of HS3. An experiment was devised to ascertain the fate of ^{32}P-labelled HS3 during the amino acid starvation period. The results of fig 11 show that when exogenous ^{32}Pi was withdrawn from cells incubated in glutamine-deficient medium, the prelabelled HS3 remained constant at its elevated level while cells not starved for glutamine retained their low basal HS3 level. Because ^{32}P-HS3 did not decrease during the next 2 hr, it can be concluded that the molecule was not being converted to some other product as would be expected if unlabelled phosphate in the fresh medium was acting as a 'chase'. Glutamine withdrawal, therefore, was stimulating HS3 synthesis. The question remains unanswered whether glutamine acts by stimulating HS3 utilisation or conversion to some other metabolite(s) because of the rapid decline in its level when glutamine was added to cells with accumulated HS3 (fig. 10a).

Figure 11: Determination of levels of ^{32}Pi-pre-labelled HS3 in CHO WT cells following the withdrawal of ^{32}Pi from the incubation medium. The growth, washing, ^{32}Pi-labelling of cells and the quantitation of HS3 were as described in legend to fig 3. ^{32}Pi-labelling was carried out for 2 hr and after which the incubation medium was completely replaced with 'cold' medium. (⊙——⊙), HS3 (minus glutamine, plus ^{32}Pi at zero time); (⊡——⊡), HS3 (plus glutamine, plus ^{32}Pi at zero time); (⊙- - - -⊙), HS3 (minus glutamine, ^{32}Pi removed at 2 hr); (⊡- - - -⊡), HS3 (plus glutamine, ^{32}Pi removed at 2 hr.

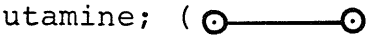

1·5
1·0
0·5
HS 3- CPM (x10⁻³)
³²Pi removed
0
1
2
3
4
Time (hr)

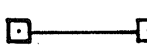
(11) Time Course Studies of the Effects of 5-Fluorouracil (5-FU) on Elevated HS3 Levels Accumulated During L-Glutamine Starvation or MTX Treatment

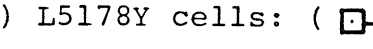
As described previously (see figs. 8, 9a and table 5), glutamine deprivation or exposure to MTX resulted in an accumulation of HS3 by CHO WT cells while 5-FU effectively antagonized the increase in HS3 induced by the glutamineless condition (see table 5). These observations, coupled with a recent report (Ullman et. al. 1978) which suggested the possible antagonistic effect of MTX on 5-FU cytotoxicity, led to a time-course evaluation of the activities of these two common antineoplastic drugs on HS3 metabolism in CHO WT and L5178Y mouse leukemia lymphoblasts. The results are illustrated in figures 12a, b and c. The addition of 5-FU to glutamine-starved CHO WT cells resulted in an immediate depletion of HS3 to control levels within 2 hr (see fig. 12a). In contrast, fig. 12b shows that the addition of 5-FU to MTX-treated cells did not elicite an immediate depletion of the HS molecule though the continued accumulation of the polyphosphorylated dinucleoside was severely restricted. A moderate decline in cellular HS3

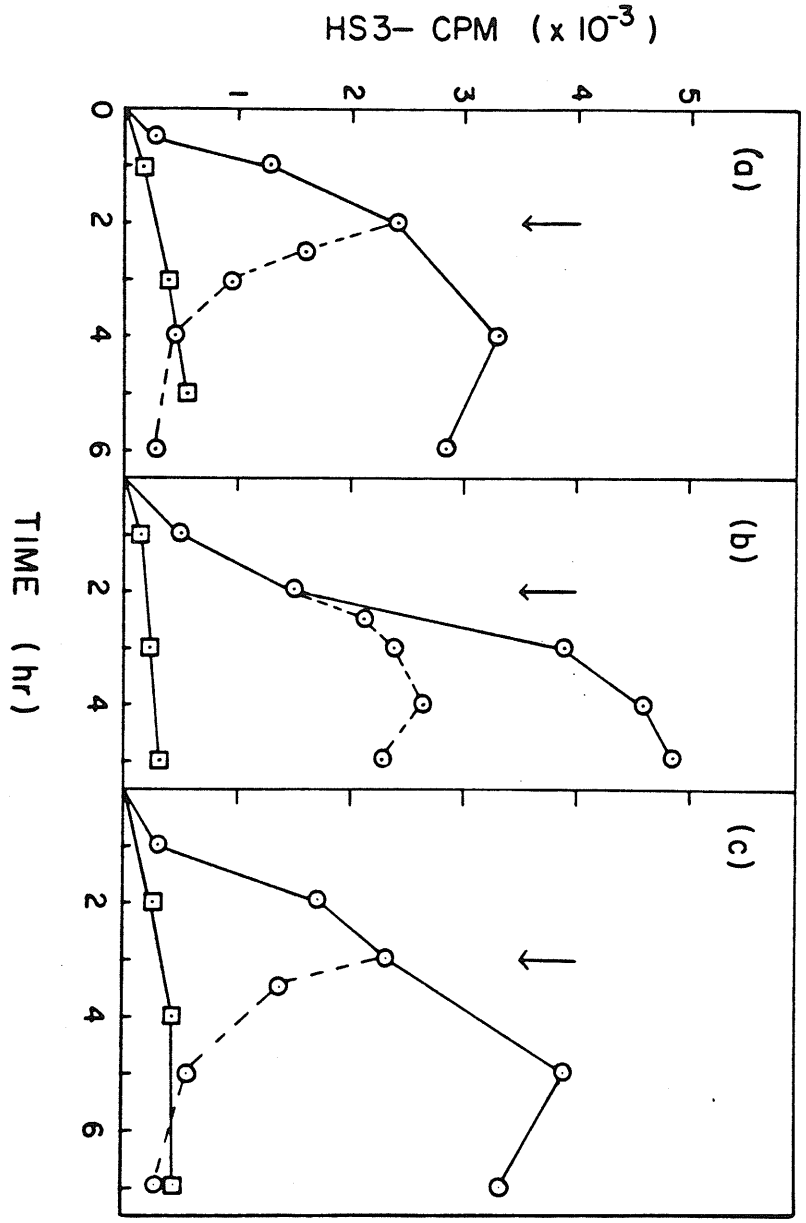
was, however, observed 2 hr following 5-FU exposure. By the third hour, the level of HS3 had decreased to 50% of non-treated cultures. When L5178Y mouse leukemia lymphoblasts were treated with 10 μM MTX for 3 hr followed by the addition of 5-FU, as illustrated in fig. 12c, the intracellular HS3 which had been accumulated was depleted to control levels within 2 hr. These results are comparable with those reported in fig. 12a. The data which have just been presented suggest that while glutamine withdrawal or MTX treatment evoked an increase in HS3 synthesis, 5-FU was effective in antagonizing this HS3 response . The mechanism(s) for this phenomenon, though intriguing, remains to be elucidated.

Figure 12: Effect of 5-FU on ^{32}P-labelled HS3 accumulated during glutamine starvation or MTX treatment using continuous ^{32}P-labelling procedure. Experimental protocol as described in legend to fig. 3 and in 'Methods'. The arrows indicate the time when 0.5 mM 5-FU was added.

(a) CHO WT cells: (), plus glutamine; ( ), minus glutamine; (), minus glutamine plus 5-FU.

(b) CHO WT cells: (), minus MTX; ( ), plus MTX (1 μM); (), plus MTX (1 μM) plus 5-FU.

(c) L5178Y cells: ( ), minus MTX; (), plus MTX (10 μM); (), plus MTX (10 μM) plus 5-FU.


HS3– CPM (x 10⁻³)
1
2
3
4
5
TIME (hr)
0
2
4
6
(a)
(b)
(c)

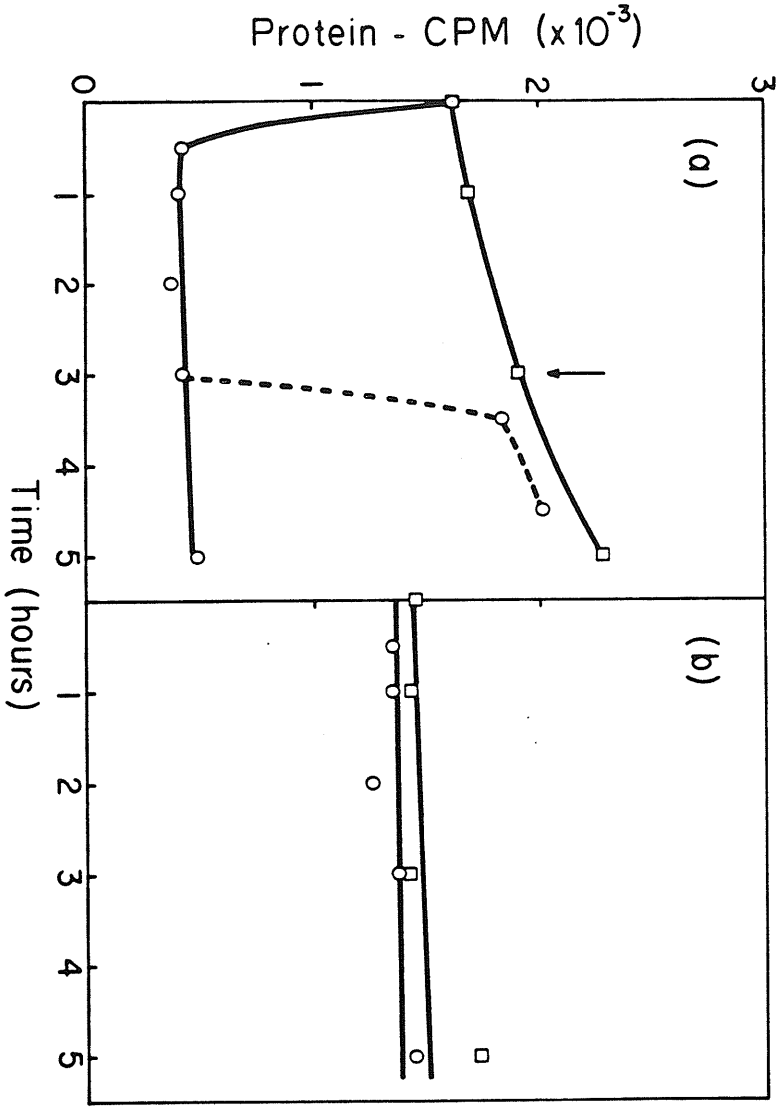
(12) Effect of L-Glutamine and Adenosine Starvation on Protein Synthesis

Bacterial ppGpp has been shown to be a potent inhibitor of *in vitro* bacterial protein synthesis (Yoshida et. al. 1972) by specifically blocking the formation of the protein synthesis initiation complex. It was therefore of interest to determine whether or not a correlation could be observed between the intracellular pool sizes of HS3 and the rate of protein biosynthesis using ^{3}H-leucine pulse labelling experiments. As shown in fig. 13a, the rate of ^{3}H-leucine incorporation by CHO WT cells was maximally inhibited within one hour following glutamine deprivation. Upon replenishment of glutamine to the starved culture, the optimal rate of protein synthesis was achieved rapidly. However, when adenosine was withdrawn from the growth medium of CHO GAT^{-}cells, the rate of ^{3}H-leucine incorporation into protein was unimpaired (fig. 13b) for at least 5 hr. Since results show (fig. 3 and table 3) that glutamine and adenosine withdrawal from the growth media of CHO WT and CHO GAT^{-}cells respectively, invariably resulted in an accumulation of HS3, protein synthesis, therefore, is apparently independent of the levels of intracellular HS3.

Figure 13: Effect of nutrient starvation and replenishment (glutamine or adenosine) on rate of ^{3}H-leucine incorporation into proteins. Cells were pulsed for 15 min with ^{3}H-leucine (3 µCi/plate) at the times indicated.

(a) CHO WT cells: (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine; (⊙- - - -⊙), glutamine (2 mM) added to glutamine-starved cultures at the time indicated by the arrow.

(b) CHO GAT^{-} cells: (⊙——⊙), minus adenosine; (⊡——⊡), plus adenosine.


Protein - CPM (x10⁻³)
0
1
2
3
Time (hours)
1
2
3
4
5
(a)
(b)

(13) Effect of L-Glutamine and Adenosine Deprivation on Rate of ^{3}H-Thymidine Incorporation Into DNA

In vitro studies (Lewis et. al. 1976; Lewis et. al. 1977) have shown that HS3 is a potent inhibitor of both ribonucleotide reductases from Achlya and CHO cells. The enzyme is involved in catalysing the reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates for DNA synthesis (Larsson and Reichard 1976). Also in Achlya, an inverse relationship between rates of DNA synthesis and intracellular HS3 levels was observed (Lewis et. al. 1976). It was therefore pertinent to determine if a similar phenomenon exists in cultured mammalian cells subjected to fluxes in nutrient availability.

Table 7a illustrates the effects of glutamine starvation and its replenishment on the rate of ^{3}H-thymidine incorporation into DNA by CHO WT cells. The rate of thymidine incorporation dropped to about 25% of control within 1.5 hr following the removal of glutamine from the growth medium. Upon readdition of 2 mM glutamine to glutamined-starved cultures, the rate of DNA synthesis

increased rapidly, reaching 75% of the control rate within 1 hr.

CHO GAT$^-$ cells were similarly affected by adenosine withdrawal (table 7b), although a marked drop in the rate of ^{3}H-thymidine incorporation into DNA was not observed before 2 hr post starvation. However, the readdition of adenosine to cells deprived of this nutrient resulted in a rapid recovery in DNA synthesis. Within 1 hr, the rate was about 90% of control.

The data presented are consistent with the results obtained from the Achlya studies (Lewis et. al. 1976). It is therefore likely that HS3 may regulate DNA synthesis in mammalian cells by exerting its effect on deoxyribonucleoside diphosphate synthesis.

TABLE 7a

Effect of L-glutamine withdrawal and replenishment on rates of ^{3}H-TdR incorporation into DNA in CHO WT cells.

Time (hr) after L-glutamine withdrawal	^{3}H-TdR incorporated into DNA expressed as % of control
0.5	57%
1.0	40%
1.5	24%
2.5	25.5%
3.5	32%
Time (hr) after addition of L-glutamine to 2.5 hr starved cells	---
1.0	75%

The cells were pulsed for 15 min with ^{3}H-TdR (3 µCi/plate; 22 Ci/m mol) at the specified intervals. For replenishment of nutrient, L-glutamine (2 mM) was added 2.5 hr after initiation of starvation. The control, expressed as 100%, is the amount of ^{3}H-TdR incorporated into DNA by non-starved cells in 15 min.

TABLE 7b

Effect of adenosine withdrawal and replenishment on rates of ^{3}H-TdR incorporation into DNA in CHO GAT^{-} cells.

Time (hr) after adenosine withdrawal	^{3}H-TdR incorporated into DNA expressed as % of control
0.5	89.7%
1.0	81.5%
2.0	30.4%
3.0	18.2%
4.0	15 %
Time (hr) after addition of adenosine to 3 hr starved cells	---
1.0	90 %

The procedure for ^{3}H-TdR (3 μCi/plate) pulse labelling was exactly as described for Table 7a. For replenishment of adenosine (0.1 mM), the nutrient was added 3 hr after initiation of starvation.

(14) Effect of L-Glutamine or Adenosine Withdrawal and Replenishment on RNA Accumulation by CHO WT and CHO GAT$^-$ Cells

The consequence(s) of amino acid withdrawal on RNA metabolism in cultured mammalian cells remains paradoxical (see 'Historical'). However since it was observed (figs. 8, 9a and b, 10a and b) that nutrient availability is closely related to changes in intracellular HS3 levels, experiments were performed to determine : (i) the effects of nutrient starvation and replenishment on RNA synthesis by CHO WT and CHO GAT$^-$ cells and (ii) if changes in RNA synthesis could be correlated with fluctuations in cellular HS3 levels.

The results in fig 14a show that total RNA accumulated , as measured by ^{3}H-uridine incorporation, was 50% of control after 5 hr of glutamine deprivation. It should be noted that uptake of uridine was not affected during this time period. Upon glutamine replenishment, incorporation of ^{3}H-uridine into RNA increased rapidly and paralleled that for control cultures. When CHO GAT$^-$ cells were deprived of adenosine, as illustrated by fig. 14b, the accumulation of RNA

was drastically reduced when compared to the controls. Upon replenishment of adenosine (0.1 mM), RNA accumulation resumed at control levels after a short lag.

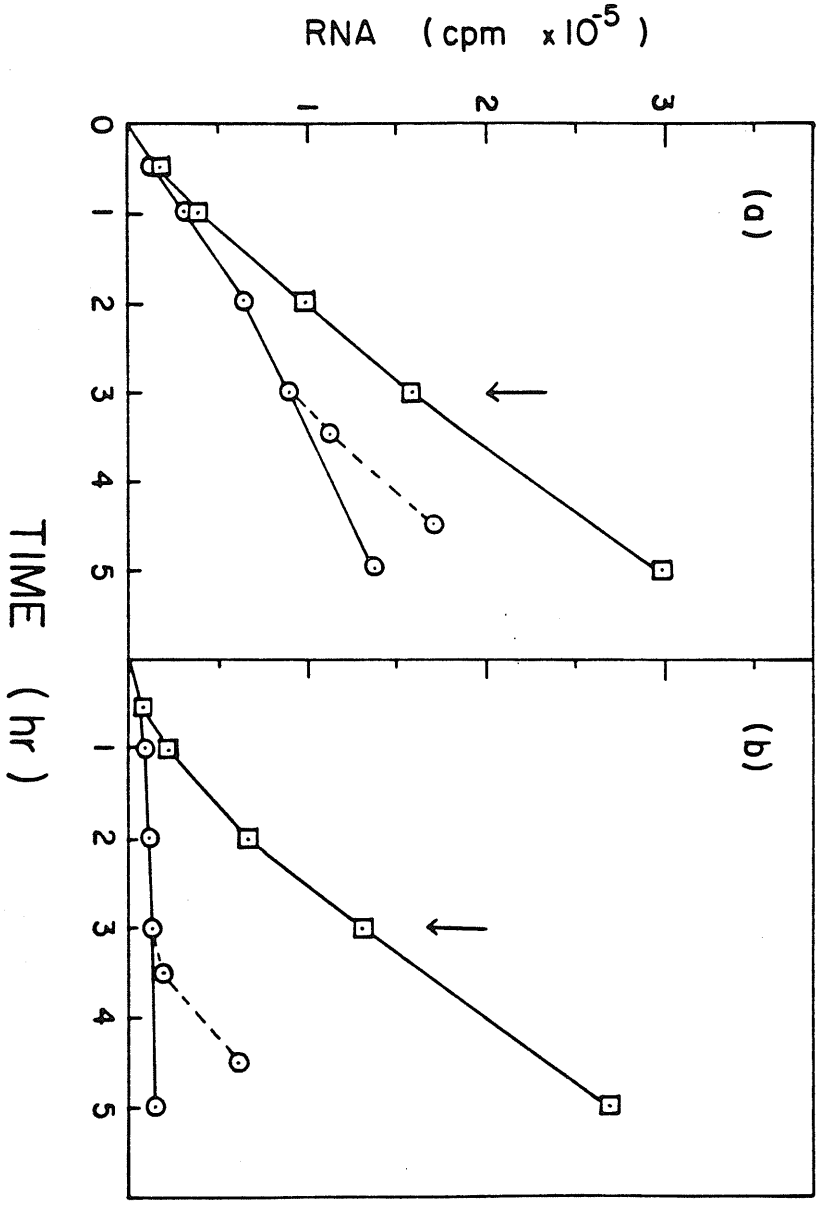
The data clearly show that there is an inverse relationship between HS3 levels and RNA synthesis, suggesting that the HS compound may be involved in the regulation of some aspect of RNA biosynthesis. Results to be presented in following sections will further support this hypothesis.

(15) Effects of L-Glutamine and Adenosine Deprivation and Replenishment on the Growth of CHO WT and CHO GAT$^-$ Cells

The data presented in the two preceding sections (13 and 14) of 'Results' showed an inverse relationship between HS3 levels and RNA and DNA synthesis. It was therefore of interest to determine if changes in growth rates during nutrient deprivation and replenishment could be correlated with the above observations.

The effects of glutamine starvation and its subsequent replenishment on the growth rates of CHO WT cells are illustrated in fig. 15a. A sharp reduction

Figure 14: Effect of nutrient starvation and replenishment (glutamine or adenosine) on ^{3}H-uridine incorporation into RNA. ^{3}H-Uridine (3 μCi/plate) was added to cells at zero time and samples taken at times indicated. (a) CHO WT cells: (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine; (⊙- - -⊙), glutamine (2 mM) added to glutamine-starved cells at the time indicated by the arrow.
(b) CHO GAT^{-} cells: (⊙——⊙), minus adenosine; (⊡——⊡), plus adenosine; (⊙- - -⊙), adenosine (0.1 mM) added to adenosine-starved cells at the time indicated by the arrow.

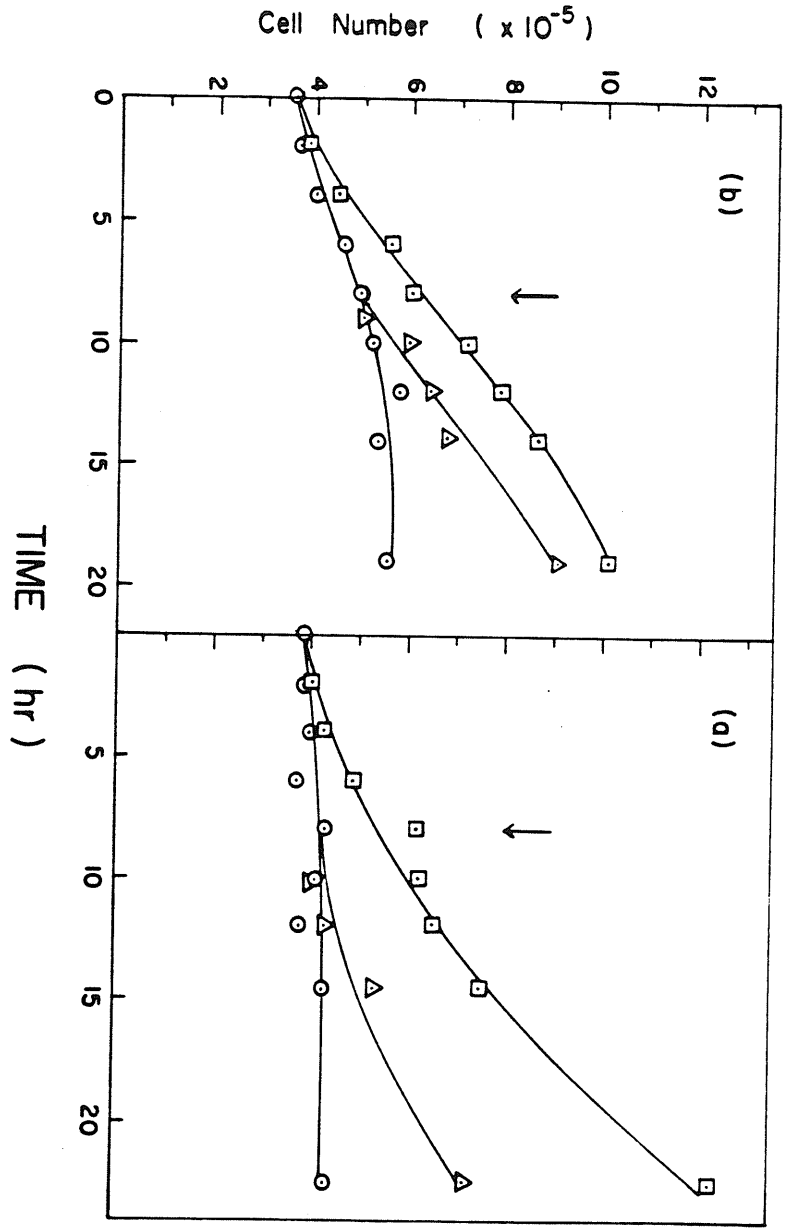

RNA (cpm x 10^-5)
1
2
3
TIME (hr)
0
1
2
3
4
5
(a)
(b)

in cell proliferation can be observed upon glutamine deprivation; cell numbers increased by only 10% in 8-10 hr after initiation of starvation and they remained constant thereafter. Upon replenishment of the cells with glutamine, the rate of growth, after a lag, increased to the rate of control cultures.

Figure 15b illustrates the results of a similar study done with CHO GAT^- cells. When starved of adenosine, the growth rate of GAT^- cells decreased rapidly and by 14-15 hr, growth had ceased completely. When the cells were re-supplemented with 0.1 mM adenosine, the growth rate increased quickly and reached that of the control culture.

Besides RNA and DNA synthesis, it is apparent now that there is also an inverse relationship between HS3 levels (figs. 10a and b) and growth rates.

Figure 15: Effects of nutrient starvation and replenishment (glutamine or adenosine) on the growth of cultured mammalian cells. Cell numbers were determined by trypsinizing the cells in each culture plate (0.05% trypsin) and counting them with a Coulter counter. The arrows indicate the time at which either glutamine (2 mM) or adenosine (0.1 mM) was added to nutrient starved cultures. The experimental procedures for growth, washing and incubating the cells were as described in 'Methods'. (a) CHO WT cells: (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine; (△——△), minus glutamine, plus glutamine. (b) CHO GAT^- cells: (⊙——⊙), minus adenosine; (⊡——⊡), plus adenosine; (△——△), minus adenosine, plus adenosine.


Cell Number (x 10^{-5})
2
4
6
8
10
12
0
5
10
15
20
TIME (hr)
(b)
(a)

(16) Effects of Achlya and CHO WT HS3 on Partially Purified CHO WT Cell DNA-Dependent RNA Polymerases

The results which have been presented (figs 10a and b, 14a and b) showed an inverse relationship between RNA accumulation and cellular HS3 level, whereby an increase in HS3 is related to a decrease in RNA synthesis and vice versa. Recently LêJohn et. al. (1978) reported that HS3 was a potent inhibitor of the enzymic activities of Achlya DNA-dependent RNA polymerases. Similar studies were performed to determine the effect of purified HS3 on the in vitro activities of mammalian DNA-dependent RNA polymerases.

DNA-dependent RNA polymerases were isolated from mid-log phase CHO WT cells grown in suspension culture. The DEAE-Sephadex A-25 chromatographic profile of the polymerases are shown in fig. 16. Peak B is RNA polymerase II, as shown by its sensitivity to 1 µg/ml α-amanitin (see table 8), while peak A, which is resistant to the bicyclic peptide, is RNA polymerase I. RNA polymerase I and II were eluted from the column at 0.14 M $(NH_4)_2SO_4$ and 0.25 M $(NH_4)_2SO_4$ respectively.

Figure 16: DEAE-Sephadex A-25 chromatography (1.5 x 6 cm column) of CHO WT DNA-dependent RNA polymerases using a linear gradient (0.04-0.5 M) of ammonium sulfate. Fractions of 0.8 ml were collected and enzymic activities determined as described in 'Methods'. Peak A is RNA polymerase I and peak B is RNA polymerase II.

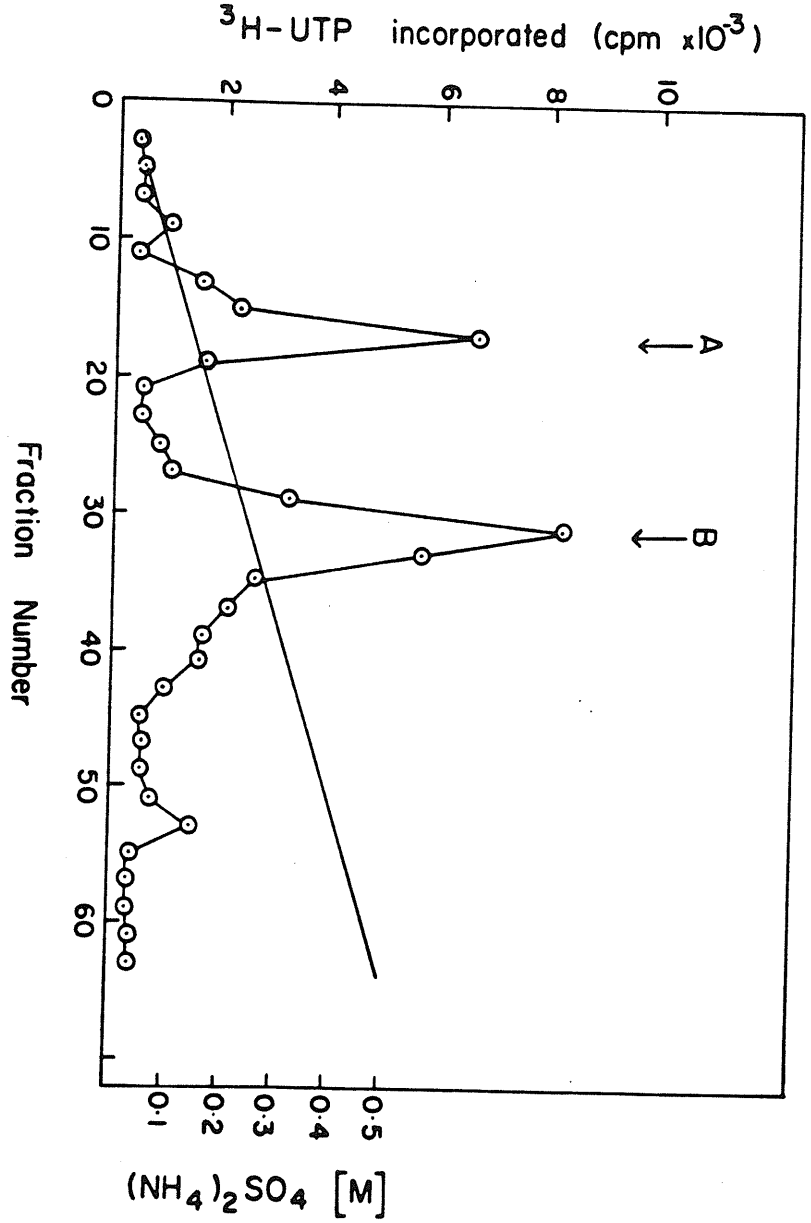

^{3}H-UTP incorporated (cpm x10^{-3})
2
4
6
8
10
0
10
20
30
40
50
60
Fraction Number
A
B
0·1
0·2
0·3
0·4
0·5
$(NH_4)_2SO_4$ [M]

Table 8 illustrates the inhibitory action of Achlya and CHO WT cell HS3 on mammalian DNA-dependent RNA polymerase activities. RNA polymerase I and II activities were inhibited by 75% and 89% respectively in the presence of 20 µg/ml of Achlya HS3.In comparison, HS3 from CHO WT cells, at a final concentration of 16.6 µg/ml, inhibited RNA polymerase I and II by 63% and 84% respectively. At higher concentrations of HS3, the activities of the polymerases were inhibited even further. In the case of RNA polymerase II, the inhibition was 95% in the presence of 41.5 µg/ml HS3.

These results support the hypothesis that HS3 may regulate RNA synthesis, possibly at the transcription level.

(17) Effect of Purified Achlya HS3 on the Incorporation of ^{3}H-UTP into RNA by Permeabilized CHO WT Cells

Since HS3 seems to inhibit the transcription apparatus of CHO WT cells in vitro, it was of interest to determine whether the effect could be manifested under in vivo or near in vivo conditions. For this

TABLE 8

Effect of Achlya and CHO WT cell HS3 on the in vitro activities of DNA-dependent RNA polymerases from CHO WT cells.

	RNA Polymerase I	RNA Polymerase II
Achlya HS3 (µg/ml)	% Inhibition	
0 + α-amanitin	96	16
0	0	0
1	15	-6
3	35	27
5	48	43
10	64	64
20	75	89
CHO WT HS3 (µg/ml)		
0	0	0
8.3	35	74
16.6	63	84
41.5	83	95

The assay procedure is described in 'Methods'. The amount of ^{3}H-UTP incorporated into TCA insoluble material in the absence of HS3 is expressed as 0% inhibition. α-Amanitin (1 µg/ml) was used to distinguish between RNA polymerase I and II . (Please see 'Results').

permeabilized cells were used to assay for HS3 effect on transcription.

The time course incorporation of ^{3}H-UTP into TCA precipitable material by permeabilized CHO WT cells, using an *in vivo* assay specific for RNA synthesis is shown in fig. 17. The incorporation of labelled UTP was linear, at least for the first hour. This time period was chosen for the subsequent studies on the effect of *Achlya* HS3 on RNA synthesis by permeabilized CHO WT cells. It was found that about 25% of the TCA insoluble material labelled in 1 hr was resistant to NaOH hydrolysis i.e. non-RNA material.

When 100 µg/ml (final concentration) of *Achlya* HS3 was added to the '*in vivo*' assay mix, the incorporation of ^{3}H-UTP into RNA by permeabilized CHO WT cells was found to be inhibited by 37%* as shown in table 9 . Higher concentrations of HS3 were not tested.

The *in vitro* (table 8) and *in vivo* (table 9) inhibitory effects of HS3 on transcription suggest that this polyphosphorylated dinucleoside is probably involved in the regulation of RNA synthesis in cultured mammalian cells.

* Actually 49.3% considering that 25% of incorporated material is NaOH-resistant

Figure 17: Time course incorporation of ^{3}H-UTP into 10% TCA precipitable material by permeabilized CHO WT cells using an *in vivo* assay for RNA synthesis; 0.9×10^{6} cells were used in the assay for each time point.

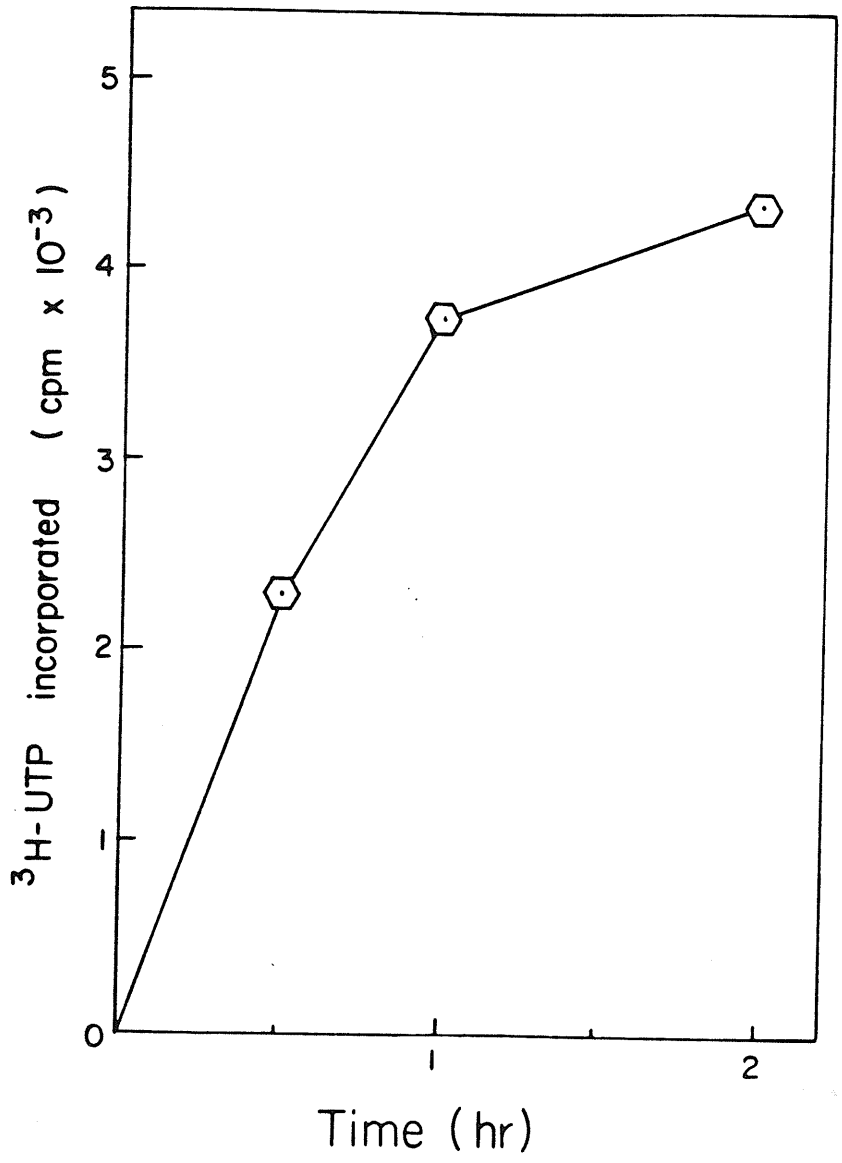

^{3}H-UTP incorporated (cpm x 10^{-3})
0
1
2
3
4
5
1
2
Time (hr)

TABLE 9

HS3 inhibition of RNA synthesis* in permeabilized CHO cells.

HS3 concentration (μg/ml)	% Inhibition
0	0
100	37 (Average of two expt.)

* ^{3}H-UTP incorporation into TCA insoluble material by permeabilized CHO cells was measured as described in 'Methods'. The amount of ^{3}H-UTP incorporated into TCA insoluble material in the absence of HS3 is expressed as 0% inhibition. A correction as described for fig. 14a & b, was made for the possible incorporation of ^{3}H-UTP into DNA (Please see pg 80 of 'Methods').

(18) Effect of Antibiotics on HS3 Biosynthesis

The preceding results suggested that nucleic acid synthesis in these cell lines suffered from nutrient deprivation, while HS3 accumulated under such starvation condition. Therefore, antibiotic inhibitors of protein and nucleic acid synthesis were used to determine what effect they might have on HS3 synthesis.

The effects of puromycin (100 µg/ml), cycloheximide (10 µg/ml) and actinomycin D (1 µg/ml) on HS3 synthesis by glutamine-deprived CHO WT cells are shown in fig. 18. Actinomycin D, was the most effective in depleting accumulated intracellular HS3. Within 1 hr after its addition, HS3 level decreased to control quantities. Both cycloheximide and puromycin were also able to lower HS3 levels, although at a much reduced rate. In the presence of glutamine in the growth medium, neither 10 µg/ml actinomycin D nor cycloheximide had any effect on the intracellular HS3 levels as shown in table 10.

Figure 18: Effect of antibiotics on accumulated ^{32}Pi-labelled HS3 during glutamine starvation of CHO WT cells. The experimental protocol was exactly as described in the legend to fig. 3. Antibiotics were added to glutamine-starved cells at the time indicated by the arrow. (⊙——⊙), minus glutamine; (⊡——⊡), plus glutamine; (●——●), minus glutamine + actinomycin D (1 µg/ml); (△——△), minus glutamine + cycloheximide (10 µg/ml); (■——■), minus glutamine + puromycin (100 µg/ml).

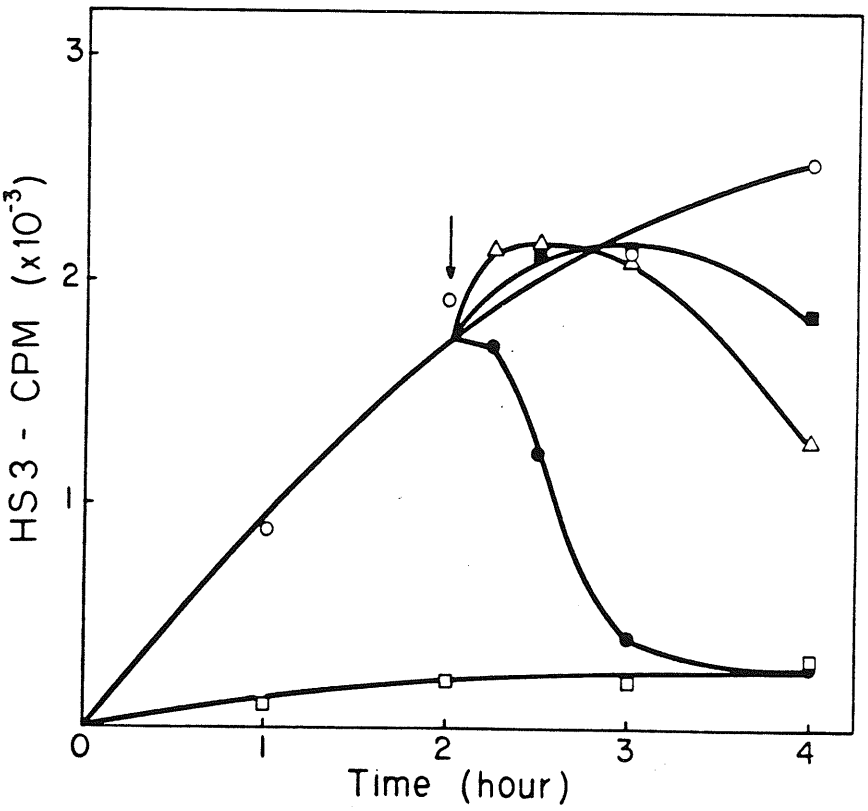

HS 3 - CPM (x10⁻³)
3
2
1
0
1
2
3
4
Time (hour)

TABLE 10

Effect of cycloheximide and actinomycin D on HS3 levels in CHO WT cells.

	HS3 (expressed as % of control)
+ glutamine	9.6
- glutamine	100.0
+ glutamine + cycloheximide	7.9
- glutamine + cycloheximide	52.8
+ glutamine	10.7
- glutamine	100.0
+ glutamine + actinomycin D	9.8
- glutamine + actinomycin D	8.3

The control, expressed as 100%, is the amount of ^{32}P-labelled HS3 present in cells after glutamine deprivation. The time of incubation was 2 hr and 3 hr for the cycloheximide and actinomycin D experiments respectively. The antibiotics, at 10 µg/ml, were added at the start of the incubation period. The experimental procedures were as described in the legend to fig. 3.

(19) Survey of HS3 Metabolism by Various Cultured Mammalian Cell Lines

A variety of cultured mammalian cell lines have been tested, in the presence or absence of 2 mM glutamine, for the production of HS3. Table 11 summarizes the results obtained, though some have already been presented in preceding sections of the 'Results'. All permanent cells lines, except CHO GAT^- cells, were found to accumulate HS3 owing to glutamine withdrawal. The reason for the lack of HS3 accumulation by CHO GAT^- cells (also see table 3) will be presented in the 'Discussion'. In contrast to the above observations, two anomalies are apparent from the results for the primary cell lines. First WI-38 cells, a cell line of fetal origin (Hayflick and Moorhead 1961) incorporated only low levels of ^{32}Pi into HS3 upon glutamine deprivation. This is illustrated by the low $\frac{HS3}{GTP}$ (cpm) ratio (table 11). Secondly, in spite of the presence of glutamine, all the rest of the primary cell lines surveyed incorporated substantial amounts of ^{32}Pi into HS3. This can be contrasted with the ^{32}P-labelled HS3 accumulated by glutamine-supplemented permanent cell lines (see HS3 percentages and

$\frac{HS3}{GTP}$ (cpm) ratios given for normal foreskin fibroblasts and CHO WT and CHO GAT^- cells). The lower $\frac{HS3}{GTP}$ (cpm) ratio for the glutamine supplemented human foreskin fibroblasts, for example, is due to a higher amount of labelled GTP as compared to the glutamine starved condition.

Owing to the unique HS3 physiological response by primary cell lines to glutamine sufficiency, it became of interest to determine if the HS3 as detected in primary cell lines by one-dimensional chromatography (table 11), is similar to the HS compound isolated from Achlya and CHO WT cells. Two-dimensional chromatography and some chemical analysis were performed. Figures 19a and 19b are two-dimensional autoradiograms of formic acid extracts from ^{32}P-labelled human foreskin fibroblasts incubated either in the presence or absence of glutamine. The $\frac{HS3}{GTP}$ (cpm) ratios of 0.72 and 1.08 for plus and minus glutamine respectively are nearly identical to those obtained from one-dimensional chromatography (table 11). Further, the two-dimensional chromatographic profile of HS3 from human foreskin fibroblasts (figs. 19a and b) is similar to that obtained for CHO WT cells (fig. 4 and table 5). Chemical analysis of HS3 isolated from Lesch-Nyhan human

fibroblasts indicate a 1:1 adenosine to uridine ratio (table 2). Thus the HS3 isolated from primary cell lines is probably similar, if not identical to the Achlya and CHO WT HS compound.

TABLE 11

Determination of ^{32}P-labelled HS3 in various mammalian cell lines cultured in the presence or absence of 2 mM L-glutamine.

	[1] HS3	
	+ glutamine	- glutamine
Permanent cell lines		
CHO WT $^-$	12.1 (0.045)*	100 (0.683)
CHO GAT $^-$	96.0 (0.049)	" (0.090)
CHO HGPRT $^-$ (YH 21)	16.7	"
CHO HGPRT $^-$/APRT $^-$ (YHD 13)	14.8	"
Balb 3T3 (mouse)	15.8	"
Balb SV 3T3 (mouse)	10.5	"
BHK 21/Cl 13	15.1	"
HeLa	24.7	"
L5178Y	41.0	"
Primary cell lines		
WI-38	[2] 90.5 (0.183)	" (0.214)
Lesch-Nyhan human fibroblast	94.3	"
Normal human fibroblast	99.0	"
Normal human foreskin fibroblast	95.0 (0.68)	" (1.07)
African green monkey kidney cells	106.0	"

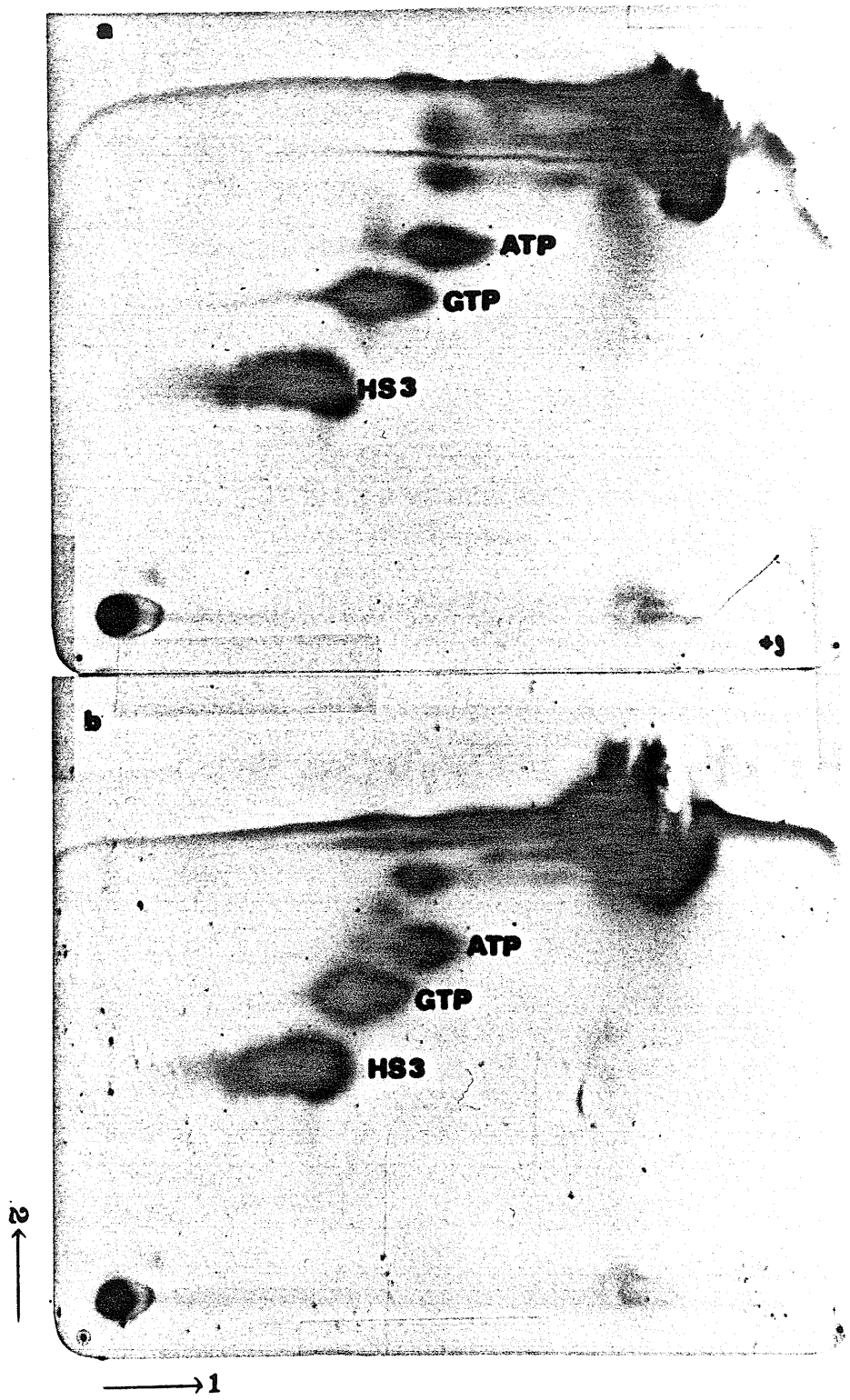
[1] ^{32}P-labelled HS3 level, after 2 hr in the absence of glutamine is expressed as 100%.

[2] An average of values after 2-5 hrs in the absence or presence of glutamine (WI-38 only).

* Values in parentheses are ratios of $\frac{HS3}{GTP}$ (cpm).

The experimental procedures and the quantitation of HS3 were exactly as described in the legend for fig. 3 and 'Methods'.

Figure 19: Two-dimensional autoradiograms of 1 M formic acid extracts of ^{32}Pi-labelled human foreskin fibroblasts (a primary cell line) cultured for 2 hr in growth medium (a) with glutamine and (b) without glutamine. The experimental procedures were exactly as described in the legend to fig 3. Two-dimensional chromatography was as described in legend to fig. 4. The ratios of $\frac{HS3}{GTP}$ (cpm) are : plus glutamine = 0.72; minus glutamine = 1.08.


a
ATP
GTP
HS3
+y
b
ATP
GTP
HS3
2
1

DISCUSSION AND CONCLUSION

(1) HS3 Synthesis by CHO WT Cells

Only one of the fungal HS compounds, HS3, was found to accumulate when glutamine was withdrawn from the growth medium of CHO WT cells (fig. 3). The absence of HS1 and HS2 cannot be completely ruled out. If they are present, their intracellular levels, at best, are not affected by glutamine availability. After a 4 hr glutamine starvation period the HS3 concentration in CHO WT cells was estimated at 0.12 mM (Lewis et. al. 1977) or 216 µg/ml cell volume assuming the MW of HS3 as 1800 (McNaughton et. al. 1978). Obviously the estimated concentration represents a minimum value as the calculation did not take into consideration the percentage recovery and the lability of the molecule during the isolation process.

Physical and chemical analyses strongly suggest that the mammalian HS3 is essentially identical to its fungal counterpart (table 2).

At no time during the course of this study was procaryotic ppGpp or pppGpp ever detected in mammalian cells. Their presence could have been easily detected chromatographically in one- and two-dimensional analyses on thin layer PEI-cellulose

plates (table 1). Furthermore, published data argue against eucaryotic cell production of procaryotic ppGpp and pppGpp (Smulson 1970; Mamont et. al. 1976; Thammana et. al. 1976; Martini et. al. 1977; Richter and Isono 1977).

(2) HS3 Accumulation: The Possible Involvement of Other Nutritional Changes Besides Glutamine Deprivation

As had been reported (fig. 3), glutamine deprivation resulted in the accumulation of HS3 in mammalian cells. However, in stringent bacterial strains, ppGpp and pppGpp were produced in response to a lack of various essential amino acids (Cashel and Gallant 1969; Cashel and Gallant 1974; Gallant and Lazzarini 1976). But this phenomenon does not exist in cultured mammalian cells with respect to HS3 accumulation. When CHO WT cells were deprived of another growth-essential amino acid, isoleucine (Ley and Tobey 1970), an accumulation of intracellular HS3 was not observed (fig. 6). This suggest that HS3 synthesis may not be related to a simple case of starvation for an essential amino acid.

Owing to the importance of glutamine as a precursor for de novo purine biosynthesis (Mahler and Cordes 1971), it became apparent that HS3 accumulation may possibly be related to a decrease in the synthesis of new cellular purines. This was substantiated through the use of two potent inhibitors of de novo purine biosynthesis. Azaserine, a glutamine analogue, specifically inhibits the amidotransferase involved in the conversion of formylglycinamide ribonucleotide to 5-aminoimidazole ribonucleotide by forming a covalently bound enzyme-inhibitor complex (French et. al. 1963). MTX is a strong reversible inhibitor of dihydrofolate reductase (EC 1.5.1.3) (Blakley 1969). The subsequent formation of 5, 10-methylenetetrahydrofolate, the active coenzyme required for purine biosynthesis (Mahler and Cordes 1971) would thus be inhibited. When these inhibitors were added individually to cells incubated even in the presence of glutamine, HS3 accumulation was evoked (table 5).

A CHO GAT^- auxotrophic mutant was subsequently utilized to further define HS3 metabolism. The de novo biosynthesis of purine nucleotides and thymidylates are blocked in this mutant owing to a defect in folate

metabolism. However its growth can be supported by supplementing ∝-MEM with adenosine, thymidine and glycine (McBurney and Whitmore 1974). Since the previous data supported the hypothesis that a stimulation of HS3 synthesis is related to a block in purine biosynthesis and possibly a lack of nucleotides, it was reasoned that the removal of adenosine from the growth supporting medium of CHO GAT^- cells should result in an accumulation of HS3 as this nucleoside is the sole source of purines. Indeed regardless whether or not glutamine and thymidine were present, there was an accumulation of HS3 in this cell line as long as adenosine was withdrawn (table 3).

Owing to this strong link between HS3 production and an aberrant de novo purine pathway, CHO WT and CHO purine salvage pathway mutants were used to determine if an exogenous purine supply could suppress the accumulation of HS3 during nutrient deprivation. The lack of hypoxanthine/guanine phosphoribosyl-transferase (HGPRT) has been associated with the Lesch-Nyhan syndrome, a human neurological disorder (Seegmiller et. al 1967). This enzyme is of utmost importance to tissues and cells which cannot biosynthesize their own

purines and are dependent on an exogenous purine source like hypoxanthine. The possible importance of HGPRT during mammalian development was discussed recently by Adams and Harkness (1976). CHO mutants, YHD 13 and YH 21, lacking HGPRT (Chasin, private communication) were found to be unable to utilize high concentrations of exogenously supplied hypoxanthine, inosine and guanosine to suppress the accumulation of HS3 during glutamine deprivation while CHO WT cells could (tables 4 and 5). The reason why inosine and guanosine could not be utilized to suppress HS3 accumulation is because there are no known eucaryotic nucleoside kinases which can catalyse the conversion of inosine and guanosine directly to their mononucleotides (Friedmann et. al. 1969). Thus these nucleosides can only be converted to mononucleotides via HGPRT after their catabolism to the respective bases by nucleoside phosphorylase (Milman et. al. 1976). This reaction is generally believed to function in vivo in the direction of nucleoside breakdown to the base and ribose-1-phosphate (Milman et. al. 1976; Li and Hochstadt 1976). Therefore, only CHO WT cells with functional HGPRT were able to utilize the bases and nucleosides.

The observed depletion of HS3 by adenine, adenosine and deoxyadenosine (tables 4 and 5) requires some clarification. Adenosine is readily converted to its nucleotide by adenosine kinase (Chan et. al. 1973), while deoxyadenosine can be catabolized to adenine by an enzyme found in mammalian tissues (Snyder and Henderson 1973). The purine base may then be salvaged by adenine phosphoribosyltransferase (APRT) to AMP. However the effectiveness of adenine in preventing HS3 accumulation by YHD 13 cells is perplexing since it is known to be deficient in both HGPRT and APRT (Chasin, private communication). But YHD 13 must possess some residual APRT activity since it could incorporate ^{14}C-adenine into various nucleotides (see fig. 7). It is pertinent to point out that all the purine compounds tested are readily utilized for adenine and guanine nucleotide synthesis by mammalian cells (fig.7, Cook and Vibert 1965; Rapaport and Zamecnik 1976). Studies by LéJohn et. al. (1978) have shown that with the exception of inosine and deoxynucleotides, those purines tested on mammalian cells (table 5) were also active in inhibiting the accumulation of HS3 by nutrient-starved Achlya. It is possible that the ineffectiveness of inosine and

deoxyadenosine in the fungal system may be due to the absence of inosine phosphorylase (Milman et. al. 1976) and the deoxyadenosine to adenine converting enzyme (Snyder and Henderson 1973).

An alternative to examining the relationship between HS3 accumulation and the supply of exogenous purine compounds was to block in vivo purine catabolism. Allopurinol, a hypoxanthine analogue commonly used in the treatment of gout, is a potent inhibitor of xanthine oxidase (EC 1.2.3.2), an enzyme which catalyses the degradation of hypoxanthine and xanthine to uric acid (Pomales et. al. 1963). The analogue was found to be marginally effective, at the concentration used, in inhibiting HS3 accumulation in glutamine-starved CHO WT cells (table 5).

It was surprising to find that the pyrimidines 5-fluorouracil (5-FU), uridine and cytidine were effective inhibitors of HS3 accumulation while thymidine was without effect (table 5; LéJohn et. al. 1978). The possible role of 5-FU in this phenomenon will be considered in detail in a later section of this 'Discussion'. It is not clear how uridine and cytidine affect HS3 accumulation. Recently Skaper et. al. (1976) reported that PRPP levels in human lymphoblasts were

decreased by glutamine withdrawal from the growth medium. Since both uridine and cytidine are potent inhibitors of aspartate transcarbamylase (Bresick 1963), these two nucleosides may spare the consumption of PRPP at the orotate phosphoribosyltransferase step of de novo pyrimidine biosynthesis. Alternatively uridine could be exerting its PRPP-sparing effect by its inhibitory action on orotate phosphoribosyltransferase activity (Hoogenraad and Lee 1974). This may be an explanation for the lower potency of cytidine (table 5) owing to the necessity of its deamination to uridine to exert its effect. Whatever the mechanism by which a PRPP-sparing effect is achieved, the greater availability of PRPP should result in an enhancement of the reutilization of purine bases for nucleotide biosynthesis. This and other proposed mechanisms related to the inhibition of HS3 synthesis by various purines and pyrimidines should be considered speculative at the moment.

LêJohn (unpublished) has suggested that (a) the HS3 molecule may act as a high-energy polyphosphate storage compound during nutritional stress; (b) the so-called inhibition of HS3 biosynthesis by various

exogenously supplied purines is in fact due to the consumption of the high energy phosphates of HS3 for nucleotide biosynthesis and (c) the constituent ADP and UDP moieties could be further used for the replenishment of all the nucleotide pools.

The data discussed support the hypothesis that a decrease in intracellular purine nucleotides may be responsible for evoking the HS3 response in mammalian cells.

(3) Effect of Glutamine Deprivation and Metabolic Inhibitors on GTP and ATP Levels

The hypothesis described above is further supported by data obtained from measurements of GTP and ATP cellular pools in cells cultured under conditions whereby HS3 is known to accumulate. Under glutamine deprivation, an increase in HS3 resulted in a corresponding decrease in GTP pools (figs. 5a and b). It is important to note that variations in the $\frac{HS3}{GTP}$ ratios were observed from experiment to experiment for each of the cell lines studied. However, the average ratio was around 0.7 to 0.6 for a 2 hr experiment. These

variations may be due to subtle differences in the nutritional state of each batch of cells owing to the possibility of differences in the amount of free amino acids in each batch of dialysed fetal calf serum. The levels of HS3 and GTP are indeed sensitive to slight fluctuations in the glutamine concentration (figs. 5a and b).

With respect to ATP pools, treatment of CHO WT cells with the de novo purine biosynthesis inhibitors, azaserine or MTX (French et. al. 1963; Blakley 1969), or glutamine deprivation resulted in a 10-30% decrease in ATP concentrations within a 2 hr period (table 6) while HS3 accumulation was rapidly occurring during this time (fig. 3, table 5). A 30% decrease in ATP concentrations, within 2 hr, has also been observed for amino acid-starved Ehrlich ascites tumour cells (Live and Kaminskas 1975; Grummt and Grummt 1976). Also ^{14}C-adenine incorporation studies, using YHD 13 cells, support these observations as the generation of ATP from very low concentrations of exogenously supplied ^{14}C-adenine was completely inhibited during glutamine starvation (fig. 7).

Thus the inverse relationship between HS3

levels and purine nucleoside triphosphate concentrations is clear. The decreased levels of these nucleotides is probably due to a combination of a lack of the generation of the triphosphates and an inhibition of the synthesis of new purine nucleotide precursors. This may be further compounded by the possibility that some of the intermediates of the de novo purine biosynthetic pathway may be shunted off for HS biosynthesis (LêJohn et. al. 1978).

(4) HS3 Metabolism: Effect of Antibiotics

Puromycin, cycloheximide and actinomycin D are all potent inhibitors of eucaryotic cell metabolism. The principal mode of action of puromycin and cycloheximide is the inhibition of protein synthesis while actinomycin D inhibits RNA synthesis by forming complexes with DNA (Gottleib and Shaw 1967; Kersten and Kersten 1974).

Recent studies have shown that ppGpp concentrations were restrained to basal levels when chloramphenicol, a procaryotic protein synthesis inhibitor, was added to amino acid starved E. coli

(Cashel 1969; Lund and Kjeldgaard 1972). In comparison, actinomycin D, cycloheximide and puromycin, in decreasing order of potency, inhibited and even lowered the HS3 accumulated during glutamine deprivation (fig. 18).

The depletion of the accumulated HS3 by these antibiotics may be related to a sparing effect of nucleoside triphosphates since it was suggested that the accumulation of HS3 may be evoked by a lack of purine nucleotides (tables 3, 4, 5 and 6). This is interesting since it has been suggested by Grummt and Grummt (1976) that the resumption of RNA synthesis following cycloheximide treatment of amino acid starved cells may be related to the expansion of ATP and GTP pools to levels comparable to non-amino acid starved cultures. Alternatively, these antibiotics may lower accumulated HS3 by either directly stimulating its degradation, inhibiting its biosynthesis, or both. This may be accomplished through the effect of these antibiotics on the synthesis of various 'enzymes' involved in HS3 metabolism. However, this is purely speculative as no supportive evidence is currently available.

One interesting feature related to these antibiotic studies has been the observation that

treatment of amino acid starved eucaryotes with protein synthesis inhibitors has consistently resulted in a restoration of RNA synthesis (Hershko et. al. 1971; Foury and Goffeau 1973; Grummt and Grummt 1976; Gross and Pogo 1976; Pogo and Zbrzezna 1978). This paradoxical response called 'phenotypic relaxedness' (Grummt and Grummt 1976) is observed only when cycloheximide is supplied to cells under conditions of metabolic stress. It is tempting to suggest that this paradoxical response may be related , in some way, to the cycloheximide effect on HS3 accumulation during glutamine deprivation and the possible role of HS3 in RNA synthesis (tables 8 and 9; fig. 14a).

(5) HS3 Levels; Elevation by MTX Treatment or Glutamine Deprivation and Depletion by 5-FU

Methotrexate (MTX), a folic acid analogue and 5-fluorouracil (5-FU), a thymine analogue are common drugs currently used either separately or in combination to treat a variety of neoplasms in man. MTX is an inhibitor of dihydrofolate reductase (Blakley 1969) and thus the addition of this drug to

cells would ultimately result in an inhibition of the biosynthesis of certain amino acids (Blakley 1969), purines (Mahler and Cordes 1971) and thymidylates (Wahba and Friedkin 1961) owing to a lack of reduced folates. High concentrations of MTX will also directly inhibit thymidylate synthetase (EC 2.1.1.45) (Borsa and Whitmore 1969), an enzyme involved in the biosynthesis of thymidine monophosphate from deoxyuridine monophosphate. The cytotoxicity of 5-FU is generally ascribed to the inhibition of thymidylate synthetase by its derivative 5-fluorodeoxyuridine monophosphate (5FdUMP)(Heidelberger 1975). In the absence of its cofactor, 5, 10 methylenetetrahydrofolate (Wahba and Friedkin 1961), thymidylate synthetase binds poorly to 5FdUMP. In its presence, the enzyme forms an irreversible complex with it and 5FdUMP (Santi et. al. 1974). Thus it became apparent that the cytotoxicity of the fluorinated pyrimidine analogue may be antagonized by MTX owing to the lack of reduced folates (Ullman et. al. 1978). The actual mechanism(s) for this antagonism remains to be resolved. It is also likely that the other nucleoside and nucleotide derivatives of 5-FU may affect other enzymic activities and RNA metabolism as well (Heidelberger 1975).

Owing to these intriguing observations, the effect of MTX and 5-FU on HS3 metabolism may be of some significance. It has been reported that glutamine deprivation or MTX treatment of CHO WT and L5178Y cells resulted in HS3 accumulation (figs. 3, 9a, 12a,b,c and table 5) and a corresponding decrease in purine nucleoside triphosphate pools of both cell lines (figs. 5 and 7; table 6; Hryniuk et. al. 1973) and also dGTP levels in L5178Y cells (Tattersall and Harrap 1973). This aspect that a decrease in purine nucleotide biosynthesis and/or pools is related to HS3 accumulation has already been discussed in previous sections.

The results showed that 5-FU, at 0.3 mM, inhibited the synthesis and even depleted the HS3 accumulated by CHO WT and L5178Y cells during glutamine starvation or MTX treatment (figs. 12a,b and c). This phenomenon may be related to a purine sparing effect previously observed for 5-FU (Tattersall et. al. 1973). This may be achieved in at least three ways: firstly, by the inhibition of thymidylate synthetase (Santi et. al. 1974) and thus sparing the consumption of dUMP, whose biosynthesis consumes ATP (Mahler and Cordes 1971); and secondly, DNA synthesis in nutrient-starved or

MTX-treated cells may be further inhibited due to the lack of dTTP, and again sparing the consumption of the other deoxynucleoside triphosphates. This has indeed been demonstrated for L5178Y cells by Tattersall and Harrap (1973). Thirdly, 5-FU itself or one or more of its metabolic derivatives may be involved in further blocking cellular RNA synthesis (Heidelberger 1975). Alternatively, rather than due to a nucleotide sparing effect, the effectiveness of 5-FU in depleting HS3 pools may be related, in some yet unknown way, to the unusual uracil-containing end of the HS3 molecule (table 2; Goh and LêJohn 1977).

One interesting observation from these studies is the fact that in CHO WT cells, the rate of HS3 depletion by 5-FU was far greater for glutamine-starved cultures when compared with MTX-treated ones (figs. 12a and b). The greater inhibition of purine nucleotide biosynthesis by MTX over glutamine deprivation is unlikely as MTX treatment, even at 10 μM, depleted ATP levels by only 12% as compared to 20% for glutamine starvation (table 6). A plausible explanation may be related to the work of Santi et. al. (1974), where they found that in the absence of reduced

folates, 5-FdUMP bound poorly to thymidylate synthetase. Thus the greater availability of reduced folates during glutamine deprivation than MTX treatment would lead to a greater purine nucleotide sparing effect by 5-FU. The fact that the depletion of HS3 in MTX-treated L5178Y cells by 5-FU was very rapid when contrasted with similarly treated CHO WT cells (figures 12b and c) may be related to the ten-fold increase in the supplemented folic acid in the growth medium of L5178Y cells than in the α-MEM used for culturing CHO cells (Fisher and Sartorelli 1964; Stanners et. al. 1971).

Though the results discussed here may have some relevance to the work reported by Ullman et. al. (1978), the physiological role(s) of 5-FU in depleting HS3 remains obscure.

(6) HS3 as a Possible Cellular Regulator: Changes in Rate of Accumulation and Pool Size of HS3

In bacteria, the deprivation of an essential amino acid evokes a cellular phenomenon known as

the 'stringent response' (Stent and Brenner 1961), whereby numerous cellular processes are blocked (see 'Stringent Response' in historical). ppGpp and pppGpp have been implicated in regulating these diverse metabolic processes. The ability of bacteria to rapidly alter their intracellular concentration of ppGpp in response to abrupt changes in the nutritional environment is a tremendous physiological asset (Cashel 1969; Gallant and Harada 1969; Gallant et. al. 1970; Fiil et. al. 1972; Stamminger and Lazzarini 1974; Cashel 1975). Data have been presented (figs. 8, 9a and b, 10a and b) demonstrating the rapid changes in HS3 metabolism during transient nutritional imbalance. The parallelism of HS3 metabolism with ppGpp metabolism suggest that this eucaryotic dinucleoside polyphosphate may be involved in some aspect of the regulation of eucaryotic cell growth.

The data from figure 11 suggest that, at least in CHO WT cells, HS3 degradation is very slow during glutamine starvation. Thus it is likely that the increase in HS3 concentrations during glutamine starvation may be the result of an acceleration in HS3 biosynthesis (figs. 8 and 9a).

There is no data, at present, to support a mechanism(s) responsible for the rapid depletion of accumulated HS3 following the replenishment of the omitted nutrient (figs 10a and b). However, the rapid fluctuation in the intracellular levels of HS3 suggest that changes in the biosynthesis and degradation and/or utilization of the different moieties of the dinucleoside polyphosphate must be operating in the eucaryotic cell.

(7) Intracellular HS3 Levels: Correlation with Protein, DNA and RNA Syntheses and Growth

(a) Protein Synthesis

Based on the data obtained (figs. 9a and b; 10a and b; 13a and b), it can be concluded that the rate of protein synthesis is independent of the intracellular levels of HS3 in mammalian cells. This is in agreement with the results reported for fungal cells by LêJohn et. al. (1975). The drastic inhibition of ^{3}H-leucine incorporation into proteins by glutamine-deprived CHO WT cells is probably the result of a deficiency in glutamine and purine nucleotides for protein synthesis rather than related to some *in vivo* effect

of HS3 on the protein synthetic process. This is in contrast to the observed correlation between an increase in intracellular ppGpp and a decrease in protein synthesis in procaryotes (Stent and Brenner 1961; Cashel 1969); and where in vitro studies (Yoshida et. al. 1972) have shown that the formation of the protein synthesis initiation complex was inhibited by ppGpp.

(b) DNA Synthesis

DNA pulse labelling studies (tables 7a and b) demonstrated an inverse relationship between the rate of DNA synthesis and HS3 concentrations (figs. 9a and b; 10a and b). A similar correlation was observed during the growth cycle of the fungus, Achlya (Lewis et. al. 1976). Further, it was reported that the activity of ribonucleotide reductase, an enzyme involved in the conversion of ribonucleoside diphosphates to their deoxy-derivatives (Larsson and Reichard 1966), decreased as HS3 levels increased (Lewis et. al. 1976). Subsequently it was shown that HS3 potently inhibited the ribonucleotide reductases from CHO WT cells and Achlya, while having

no significant effect on the E. coli enzyme (Lewis et. al. 1976; Lewis et. al. 1977). The inhibition by HS3 was found to be non-competitive with respect to the substrates (Lewis et. al. 1977). The pool sizes of the various deoxynucleoside triphosphates in a variety of cell types have been shown to be very small and without replenishment are sufficient to support DNA synthesis for only short periods of time (Reichard 1972; Walters et. al. 1973). Therefore, the rapid decline in DNA synthesis during glutamine (Ley and Tobey 1970; Pardee 1974; table 7a) or adenosine (table7b) deprivation may be related to a self-imposed starvation of cellular deoxyribonucleotides by the action of HS3 on the ribonucleotide reductase.

(c) RNA Synthesis

Stable RNA accumulation by bacteria during the 'stringent response' is severely restricted (see 'Stringent Response' in Historical). Parallel studies have been employed to determine if a similar phenomenon occurs in mammalian cells during metabolic stress. However the results reported (see 'Mammalian Cells:

RNA Biosynthesis and Amino Acid Withdrawal' in Historical) does not allow a definite conclusion to be reached regarding the biochemical consequences of amino acid withdrawal in cultured mammalian cells. It is likely that a factor contributing to these conflicting results is the use of different starvation protocols (eg. different amino acids, different growth media, length of starvation, the use of non-dialysed serum in some cases etc.).

Studies with CHO WT and CHO GAT$^-$ cells showed that ^{3}H-uridine incorporation into RNA during nutrient starvation was inhibited, but resumed without any appreciable lag at control levels upon nutrient replenishment (figs. 14a and b). It is pertinent to point out that the decrease in ^{3}H-uridine incorporation into RNA during nutrient withdrawal was not the result of a decrease in uridine uptake. Similarly, the uptake of uridine by Achlya (LēJohn et. al. 1978) and human liver cells (Bolcsfoldi et. al. 1971) was also unaffected during glutamine deprivation.

Because of these in vivo observations, in vitro studies were performed to determine what effect isolated HS3 has on the activities of the various DNA-dependent RNA polymerases from CHO WT cells.

The isolation procedure of these DNA-dependent RNA polymerases from CHO WT cells, as described in 'Methods', was essentially according to the Young and Whitely method (1975). The salt concentrations at which RNA polymerases I and II were eluted out (fig. 16) are in agreement with those isolated from rat liver (Lindell et. al. 1970). Peak B was identified as RNA polymerase II, as shown by the enzyme's sensitivity to its specific inhibitor, α-amanitin (Lindell et. al. 1970). RNA polymerase III, which is eluted by 0.45 M ammonium sulfate (Young and Whitely 1975) was found in very small quantities. RNA polymerase I is implicated in the synthesis of ribosomal RNA while RNA polymerase II is generally believed to be involved in the synthesis of heterogenous nuclear RNA, part of which is probably precursor messenger RNA (Chambon 1975). RNA polymerase III probably catalyses 5S and pre-4S RNA syntheses (Chambon 1975).

Interestingly, both Achlya and mammalian HS3 were found to be equally potent in inhibiting, in a concentration dependent manner, the in vitro activities of RNA polymerase I and II from CHO WT cells (table 8). Studies with Achlya RNA polymerases have shown a similar

HS3 inhibitory effect (LeJohn et. al. 1978). The mechanism of the *in vitro* inhibition of eucaryotic RNA polymerases by HS3, however, remains to be elucidated.

These *in vitro* studies were extended to a 'whole-cell' nucleotide-permeable system (Lewis et. al. 1978) used in conjunction with an assay specific for RNA synthesis (Castellot et. al. 1978). HS3 was also found to be a fairly potent inhibitor of RNA synthesis in this intact, permeabilized mammalian cell system (table 9). The concentration of HS3 used in this inhibition study was only approximately one-half its estimated physiological concentration in 4 hr-glutamine-starved CHO WT cells (see 'HS3 Synthesis by CHO WT Cells' of 'Discussion'). The inhibitory action of the fungal and mammalian HS3 on mammalian DNA-dependent RNA polymerases is another example of the similarity between the HS3 molecules from both eucaryotic organisms (table 2).

Based on these *in vitro* and *in vivo* data, it is suggestive that HS3 may be of physiological importance in regulating some aspect of RNA synthesis in eucaryotes.

(d) Growth

Since it was observed that the biosynthesis of nucleic acids in CHO cells was severely restricted on nutrient starvation, experiments were performed to determine what effect nutrient starvation might have on growth. It was observed that growth was severely restricted on glutamine (fig. 15a) and adenosine (fig. 15b) starvation in CHO WT and GAT^- cells respectively. The data are consistent with those previously reported by other investigators (Eagle et. al. 1955; Ley and Tobey 1970; McBurney and Whitmore 1974). The residual increase in cell numbers (figs 15a and b), despite nutrient deprivation and the accompanied inhibition of RNA and DNA synthesis may be explained by the fact that those cells in the non-synchronous cell population which are between the end of G_2 and M phases of the cell cycle (Howard and Pelc 1953) may still escape the block and proceed through mitosis. This is possible since DNA synthesis, which is restricted to the S phase, and RNA synthesis are essentially absent during late G_2 and mitosis (M phase) (Mitchison 1971).

It is apparent that glutamine deprivation of

CHO WT cells is more effective in inhibiting growth when compared to adenosine starvation of CHO GAT$^-$ cells. This may be related to the fact that protein synthesis, which continues unabated during mitosis in Chinese hamster cells (Taylor 1960), was strongly inhibited in glutamine-starved CHO WT cells while remaining unimpaired in adenosine-deprived CHO GAT$^-$cells (figs. 13a and b).

(8) Possible Effects of HS3 and Nucleoside Triphosphate Levels on Nucleic Acid Synthesis

Results have been presented which showed that during specific nutrient deprivation, a high intracellular HS3 level is associated with decreased purine nucleoside triphosphate pools, inhibition of growth and the reduction of RNA and DNA synthesis. However, what is difficult to resolve, based on the available data, is whether HS3 or ATP and GTP levels are directly involved in regulating, in vivo, RNA and DNA synthesis through their effects on the DNA-dependent RNA polymerases and the ribonucleotide reductase during nutrient deprivation. On the one hand, both ATP and GTP pools were found to decrease during glutamine starvation;

a 2 hr starvation period led to a 20% decrease in ATP levels (table 6). Thus the lowering of these essential nucleotides may be directly responsible for the observed inhibition of RNA and possibly DNA synthesis.

However Jolicoeur and Labrie (1974) reported that during a 2.5 hr glutamine starvation of Landschutz ascites cells, only 45S pre-ribosomal RNA synthesis was preferentially inhibited (about 60%) while 4S, 5S and polysomal messenger RNA synthesis was not significantly affected. This observation still does not exclude the possibility that separate nucleoside triphosphate pools for ribosomal and messenger RNA synthesis exists (Grummt and Grummt 1976). As there is no evidence to support this hypothesis, the in vitro effect of HS3 in inhibiting RNA polymerase I activity may therefore be of physiological importance. On the other hand, the significance of the inhibition of RNA polymerase II by HS3 (table 8) is unclear as in vivo studies (Jolicoeur and Labrie 1974) demonstrsted that messenger RNA synthesis was not affected by glutamine starvation. It is possible that in vivo, HS3 may have a quantitatively different effect on RNA polymerase II activity during metabolic stress. However this is

purely speculative and must await other *in vitro* studies, for example using native DNA and DNA of known coding function as templates and the measurement of the rate of biosynthesis of specific messenger RNAs.

The possible effects of decreases in ATP and GTP pools on DNA synthesis during nutrient deprivation is obscure. It has been suggested (Elford et. al. 1970; Cory and Whitford 1972) that ribonucleotide reduction is the rate-limiting step for DNA synthesis. The substrate specificity and the rate of reaction of ribonucleotide reductase are regulated in a complex, allosteric fashion by the levels of ATP and other deoxyribonucleoside triphosphates (Reichard 1972; Elford 1972). Also dATP acts as a general feedback inhibitor of the enzyme (Larsson and Reichard 1966). It would be futile to attempt to speculate on the consequences of decreased cellular ATP and GTP contents on the ribonucleotide reductase activity since not much information is available with regard to the absolute pool sizes of the various ribo- and deoxyribonucleotides in cells under conditions which would result in HS3 accumulation. What is clear, however, is that *in vitro* HS3 when compared with dATP was found to be a more potent inhibitor of the reductase (Lewis et. al. 1977).

(9) Glutamine Availability and HS3 Metabolism by Various Cultured Mammalian Cell Lines

Glutamine withdrawal studies pertaining to HS3 metabolism by various CHO cell lines were extended to other cultured mammalian cell lines. In all cases (table 11) only one of the fungal polyphosphorylated dinucleosides (McNaughton et. al. 1978), HS3, was detected. The data suggest that (a) the existence of HS3 is widespread and probably exists in most, if not all,cultured mammalian cell lines and (b) the absence of HS1 and HS2 in mammalian cells may be related to an intrinsic physiological difference between them and fungal cells; namely that all three HS compounds may be somehow involved in the fungal sporulation process (LēJohn et. al. 1978).

The removal of glutamine from the growth medium resulted in an accumulation of HS3 by all but one of the permanent cell lines. The failure by CHO GAT^- cells to accumulate HS3 is most likely due to the presence of adenosine in its growth medium. The implications of this observation have already been discussed. The primary cell lines with the exception

of WI-38, exhibited a similar physiological response as permanent ones in that they accumulated HS3 upon glutamine starvation (table 11).

The lack of ^{32}Pi labelled HS3 in WI-38 cells even after 5 hr of glutamine deprivation is perplexing because there was a significant incorporation of ^{32}Pi into other intracellular nucleotides. It is not clear whether this phenomenon manifested by WI-38 cells is related to their limited in vitro proliferative potential and embryonic origin (Hayflick and Moorhead 1961; Hayflick 1965), since none of the other cell lines surveyed possess both of these characteristics. Alternatively, it may be possible that glutamine sufficiency is attained by the rapid induction of glutamine synthetase (EC 6.3.1.2). However this appears unlikely considering the short period of starvation (2 hr). Besides, its been reported that glutamine-starved WI-38 cells did not resume growth even when a high level of glutamine synthetase was induced (Viceps and Cristofalo 1975). A survey of other primary embryonic cell lines should suggest whether or not the lack of an accumulation of ^{32}Pi labelled HS3 during glutamine deprivation is a characteristic of such cells.

Another interesting observation was the fact that during glutamine sufficiency all the primary cell lines tested, with the exception of WI-38, were found to incorporate similar high amounts of ^{32}Pi into HS3 when compared with the glutamine starvation condition (table 11; figs 19a and b). The absence of the phenomenon just described in permanent cell lines may be the result of some transformation characteristic(s) of such cell lines (see 'Growth Requirements and Characteristics of Cultured Mammalian Cells' in Historical). For example in their altered serum and nutrient requirements (Paul et. al. 1971; Rudland et. al. 1974) or changes in the composition of the proteins and morphology of their plasma membrane (Robbins and Nicolson 1975; Hynes 1976). However mouse 3T3 and BHK 21/Cl 13 cells, for example, other than being permanent cell lines (Aaronson and Todaro 1968; Macpherson and Stoker 1962) exhibit very few, if any, of the characteristics of the transformed state.

The two phenomena discussed, with regard to HS3 metabolism in primary cell lines are interesting problems. Further careful analysis will be required to determine the significance of these observations. For example it would be highly informative if the subcellular localization and concentration of HS3 in mammalian cells could be quantitated. At least in

the fungus Achlya, HS3 has been found to be associated with a proteoglycan located on the cell wall-membrane complex (Cameron and LēJohn 1978). The HS molecule is probably synthesized at the cell membrane and, depending on the growth condition, can apparently be translocated into the cell or excreted into the medium (LēJohn, unpublished data).

Since it was found (table 2) that HS3 contains adenine, uracil, pentose and hexose sugars, glutamate and phosphates, attempts to label the HS molecule with ^{3}H and ^{14}C precursors of de novo purine and pyrimidine pathways and the nucleosides and bases themselves have been without success. This may be due to the dilution of the supplemented radioactive precursors by the 'rich' medium in which the cells are cultured in. LēJohn (unpublished data) has successfully labelled Achlya HS3 using similar but high specific activity precursors. The labelling patterns of HS3 with the HS3 molecule precursors are similar to those achieved with ^{32}Pi (LēJohn, unpublished data).

Conclusion

One of the three polyphosphorylated dinucleosides (McNaughton et. al.), HS3, has now been detected in all mammalian cell line tested. The results which have been presented are consistent with two hypotheses:

(1) HS3 accumulation, owing to an increase in synthesis, in cultured mammalian cells is in some yet unknown way intimately linked with a deficient supply of purine nucleotides and/or its precursors.

(2) HS3 may be physiologically important in regulating eucaryotic RNA and DNA metabolism through its effect on the activities of the DNA-dependent RNA polymerases and ribonucleotide reductase.

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