Genetical and Biochemical Evidence that a Novel Dinucleoside Polyphosphate Coordinates Salvage and De Novo Nucleotide Biosynthetic Pathways in Mammalian Cells.

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Summary: Studies with 'wild type' Chinese hamster ovary cells and mutant derivatives defective in purine salvage and de novo nucleotide biosynthesis pathways have brought to light the possibility that an unusual dinucleoside polyphosphate, HS-3 (see appendix) is a crucial regulator of these two pathways. Three antitumour drugs, methotrexate, 5-fluorouracil and azaserine as well as L-glutamine, purines and pyrimidines were used to define the loci of HS-3 metabolism. Wild type and salvage pathways mutants accumulated HS-3 in the absence of glutamine. De novo pathways mutant accumulated HS-3 only when purine was absent. Depletion of HS-3 was induced in wild type and de novo mutant cell lines by purine compounds. Salvage pathways mutants did not cause depletion of HS-3 when supplied with purines or pyrimidines, except 5-fluorouracil. Data indicate that HS-3 is probably synthesised when an early step in purine nucleotide synthesis is blocked and depleted when the salvage pathways are operative. HS-3 may be an important factor in certain diseases involving nucleotide metabolism.

Introduction: Amethopterin (methotrexate) a folic acid analogue is a strong inhibitor of dihydrofolate reductase (EC 1.5.1.3) and probably of thymidylate synthetase (EC 2.1.1.b) (1, 2). 5-Fluorouracil and 5-fluorodeoxyuridine inhibit thymidylate synthetase (3, 4). Mammalian cells treated with both drugs should have their de novo nucleotide biosynthesis pathways seriously inhibited. When L-glutamine specifically is omitted from the growth medium of Chinese hamster ovary cells, the cell cycle is arrested at G_1. Isoleucine limitation also contributes to this cell cycle change (5). The mechanism is not understood.

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Abbreviations: $HGPRT^-$; hypoxanthine-guanine phosphoribosyltransferase-deficient
$APRT^-$; adenine phosphoribosyltransferase-deficient
GAT^- ; glycine, adenine and thymidine-requiring
CHO ; Chinese hamster ovary
5-FU ; 5-fluorouracil

Recently, we showed that a set of unusual dinucleoside polyphosphates HS-3, 2 and 1* present in abundance in fungal cells (6) inhibited DNA-dependent RNA polymerases (7) and ribonucleoside diphosphate reductases of mammalian and fungal cells (8). We also now know that HS-1 potently inhibits unresolved mixture of DNA polymerases of the fungus Achlya (unpublished data of L.E. Cameron and H.B.L.). All 3 HS compounds exist in mammalian cells used in this study and one, HS-3, has been partially characterised (to be published).

This communication provides genetic and biochemical evidence that links HS-3 with the coordinate regulation of de novo and salvage nucleotide biosynthesis pathways.

Materials and Methods:

Biological materials. All of the Chinese hamster ovary cells used in this study were generously provided by Dr. J.A. Wright of this university. His sources of the cells are: CHO derivative of the original CHO-K1 of Puck et al., (9), and GAT^-, a glycine, adenine and thymidine-requiring derivative of CHO-K1 were from the Ontario Cancer Institute, Toronto. GAT^- has been characterised by McBurney & Whitmore (10). The purine salvage mutants, CHO-K1 ⟶ YH21 ($HGPRT^-$) and CHO-K1 ⟶ YHD13 ($HGPRT^-/APRT^-$) were originally from Dr. L. Chasin of Columbia University. Dr. Rose Sheinin of the Ontario Cancer Institute, Toronto, kindly provided mouse fibroblast Balb 3T3 and simian virus 40 (SV40) transformed Balb 3T3 cell lines.

Growth conditions. The mammalian cells were routinely grown in α-minimal essential medium supplemented with either 10% dialysed or undialysed foetal calf serum (v/v) (with the vital nutrients where mutants were used) in 5% CO_2 atmosphere at 37°C. α-Minimal essential medium was obtained from Flow Laboratories Inc.

Isolation of HS-3. The procedure outlined (8) for isolation and purification of HS-3 from the fungus Achlya was used successfully for extraction of the compound from wild type CHO cells.

Results

Definition of HS-3 metabolism through mutants

The typical chromatographic behaviour of HS-3, 2 and 1 on polyethyleneimine (PEI) cellulose thin layer sheets using the solvent system of Cashel (11) but with the pH at 3.65 is shown in the autoradiogram of Fig. 1, frame IA. HS-2 and HS-1 are present in very low quantities in the acid extract of mammalian cells in contrast to fungal cells (6).

When the mammalian cells were transferred from a normal growth medium to one lacking L-glutamine, HS-3 started to accumulate within minutes. The results shown in frames IA and IB of Fig. 1 are for HS-3 levels in wild type CHO cells starved of glutamine for 2 h (frame IA) and unstarved (frame IB). A similar result was obtained for the purine salvage pathways mutants YH21 and YHD13, 3T3 and SV40 3T3 mouse fibroblast cell lines but not the CAT^-

*see appendix for a correction note on their characterisation.

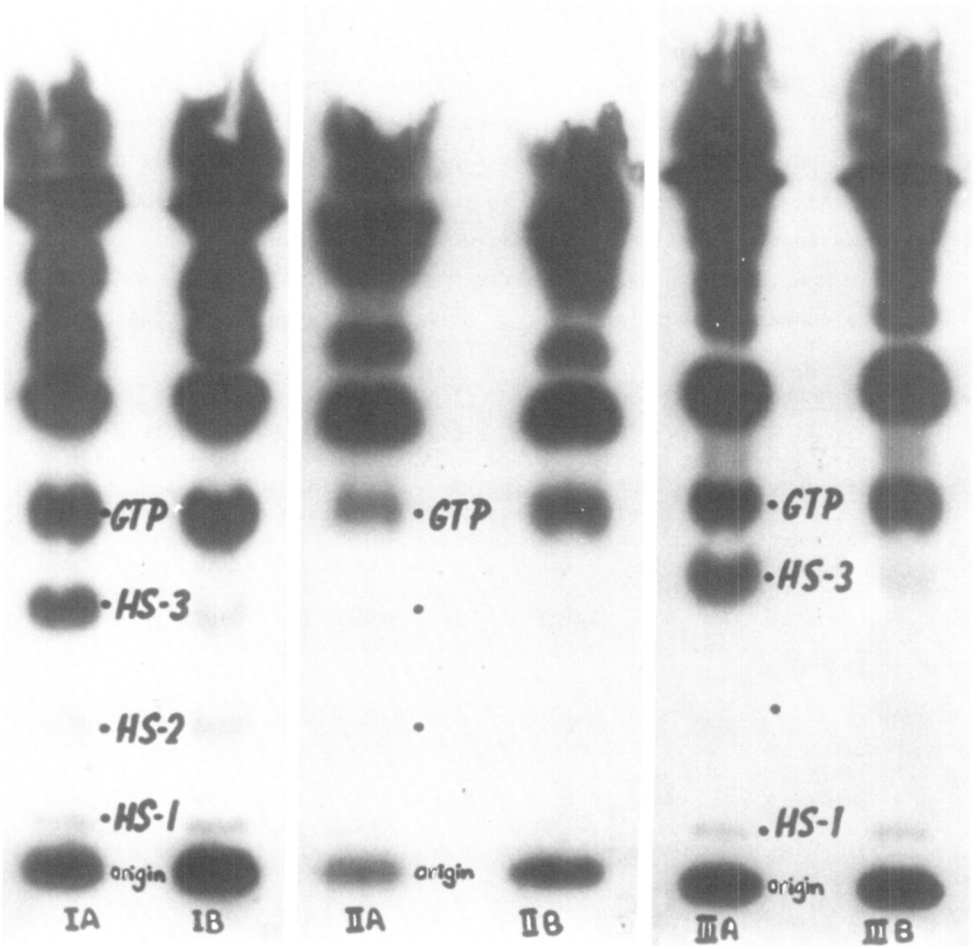

Fig. 1. Autoradiograms of 1M formic acid extracts of wild type CHO cells (left and middle panels) and YHD13 purine salvage pathway mutant (right panel) chromatographed on PEI cellulose thin layer sheets as described (6).

The cells were grown in Brockway bottles to monolayer stage then trypsinised with 0.05% trypsin for 5 min. They were then collected by centrifugation, washed with prewarmed sterile 0.9% NaCl and resuspended in fresh growth medium. The cells were replated in falcon plastic petri dishes and grown to a density of 0.5 - 0.6 x 10^6 cells/plate in 24 h. The growth medium was removed, the cells attached to plates were washed gently with prewarmed 0.9% NaCl, twice, then covered with 3 ml of the appropriate incubation medium containing 0.035 µg (0.35 mCi) $^{32}P_i$. The medium itself contained about 12 µg unlabelled P_i/ml/plate. To one culture of wild type CHO cells was added 2 mM L-glutamine (IB) and the other was glutamine-free (IA). The third culture contained 0.3 mM hypoxanthine without glutamine (IIA) and the fourth, both glutamine and hypoxanthine (IIB). The YHD13 mutant was fed with 0.3 mM hypoxanthine in the absence of glutamine (IIIA) and in the presence of glutamine (IIIB). The cells were incubated under normal growing conditions for 2 h, the media removed, then extracted directly on the plates with 0.2 ml of cold formic acid at 0° for 30 min. Ten microlitres of the acid extract was chromatographed on PEI cellulose and autoradiograph prepared as shown. The

mutant which produced HS-3 only upon adenine starvation. The importance of glutamine, essential for de novo nucleotide biosynthesis, in suppressing the synthesis of HS-3 was therefore clear. The purine salvage pathways mutants have intact de novo pathways and synthesise HS-3 when these pathways are blocked by glutamine withdrawal.

An interesting development was that, by contrast, a variety of purine and pyrimidine compounds stimulated the rapid depletion of HS-3 in all cell lines. Purines were the most effective. The autoradiograms of Fig. 1, frames IIA and IIIA, show what happens to the pool of HS-3 when hypoxanthine was fed to wild type CHO (frame IIA) and to the double mutant YHD13 ($HGPRT^-$ and $APRT^-$) (frame IIIA). HS-3 was totally depleted in wild type cells but not in the salvage pathway mutant by hypoxanthine. Therefore, the utilisation of HS-3 involved salvage path metabolism. GAT^- mutant behaved as wild type in base-induced depletion of HS-3 built up upon adenine starvation. The illustrations of frames IIB and IIIB show that glutamine suppressed HS-3 production in wild type CHO and YHD13 mutant independently of hypoxanthine action.

A comprehensive summary of the relative effectiveness of various purine and pyrimidine compounds as well as azaserine and methotrexate in altering HS-3 levels under different nutritional conditions is given in table 1 for all cell lines. These results are better appreciated if examined in 3 categories. These are: (i) CHO-K1 cells with intact salvage and de novo pathways of nucleotide biosynthesis; (ii) CHO-K1 (YH21 and YHD13) cells with defective purine salvage pathways but with intact de novo pathway; and (iii) CHO-K1 (GAT^-) cells with defective de novo but intact purine salvage pathway.

The values presented in table 1 represent the relative quantities of HS-3 in the cells 2 h after feeding the compound specified. According to the expressions given in the footnote to the table, values of 1.0 on the nil column signify that HS-3 produced in the presence and absence of L-glutamine (there is a 10-fold difference) is taken independently as 100% to serve as control. Therefore, after a particular compound was added, if the HS-3 level remained as 1.0 two hours after, the compound was ineffective. If significantly (± 33%) greater or less than 1.0, it was effective. Thus, in the wild type cell,

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relative positions of the unusual polyphosphorylated dinucleosides given the pseudonyms of HS-3, HS-2 and HS-1 are marked and the dot penciled in give the approximate positions of these HS compounds and GTP with respect to the origin. As much as 1 mM hypoxanthine has been used in the absence of glutamine to depress HS-3 level in the salvage pathways mutant without any success. The PEI cellulose plates were exposed to Kodak RP-14 Royal X-Omat films for about 24 h before developing. We note that $^{32}P_i$ was diluted about 1000-fold during the incubation.

Table 1. Influence of purine and pyrimidine compounds, methotrexate and azaserine on the synthesis and utilisation of HS-3 in a variety of mammalian cells incubated in the presence and absence of L-glutamine.

CHO cells (wild type and mutants) and mouse fibroblast 3T3 cell lines were plated in Falcon petri dishes and grown for 24 h to densities between 0.5 - 1.0 x 10^6/plate before use. At the start of the experiment, the spent growth medium was aspirated, the cells washed twice with pre-warmed test medium, then incubated in 3 ml of the appropriate test medium containing ^{32}P-orthophosphate and supplemented with 10% dialysed foetal calf serum. At the end of the incubation period (2 h), the cells were washed with ice-cold 0.9% NaCl, extracted with 0.2 ml of 1 M HCOOH at 0° for 30 min and the acid extract chromatographed on polyethyleneimine (PEI) cellulose thin layer sheets by previously described methods (6). The regions corresponding to HS-3 and GTP identified by comparison with authentic samples obtained from the fungus, Achlya, were cut out after autoradiography and radioactivity determined. All incubations were done in duplicate. The results represent averages of 2 to 3 experiments.

	Relative amounts HS-3 in cells			
	CHO-K1*	YH21	YHD13	GAT⁻
Compound added[a]	(+ : -)gln[b]	(+ : -)gln	(+ : -)gln	(+ : -)gln
nil	(1.0 : 1.0)‡	(1.0 : 1.0)	(1.0 : 1.0)	(1.0 : -)
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)	(0.23 : -)
adenosine	(0.67 : 0.08)			
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)	(1.00 : -)
thymine	(0.96 : 1.01)			
uridine	(0.92 : 0.35)		(1.00 : 0.40)	
5-fluorouracil	(0.90 : 0.11)		(0.77 : 0.17)	
azaserine	(1.70 : 1.33)			
methotrexate	(2.32 : -)		(1.8 : -)	

*Derivative of CHO-K1 (see ref. 9)

[a]Compounds added at 0.3 mM during 2 h incubation except methotrexate at 1 μM and adenine and thymidine at 0.1 mM for GAT⁻ cells.

[b]In the presence of glutamine (gln) the level of HS-3 dropped to about 13% in CHO-K1, 16% in SV40 3T3 and 21% in 3T3 cell lines compared to the control.

‡(+) = $\frac{\text{(cpm HS-3 in cells + gln + compound)}}{\text{(cpm HS-3 in cells + gln)}}$; (-) = $\frac{\text{(cpm HS-3 in cells - gln + compound)}}{\text{(cpm HS-3 in cells - gln)}}$

CHO-K1, purine compounds decreased the cellular pool of HS-3 7-12 fold when L-glutamine was absent and slightly or not at all when glutamine was present. Pyrimidine compounds except 5-FU, were less effective than the purine compounds in stimulating the depletion of HS-3. By contrast, azaserine and methotrexate

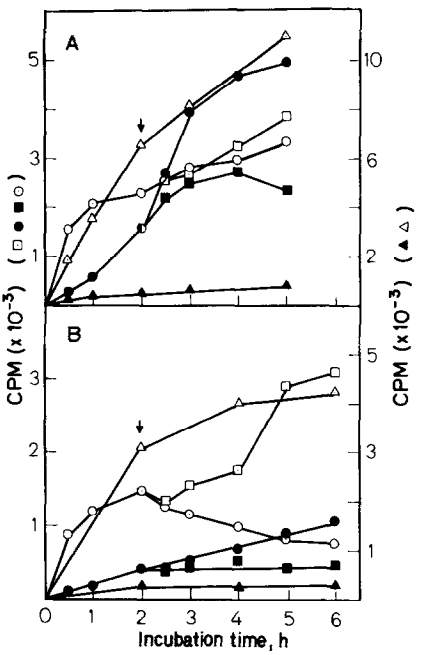

Fig. 2. The effects of methotrexate (MTX) and 5-FU on the synthesis of HS-3 and GTP in wild type (Fig. 2A) and YHD13 mutant (Fig. 2B) CHO cell lines.

The experimental procedure was as described in the legend to Fig. 1. After autoradiography, the areas on the PEI cellulose plates corresponding to HS compounds and GTP were cut and the radioactivities determined in a Beckman LS-230 liquid scintillation counter. In these experiments, 1 μM methotrexate was added at zero time to one set of cultures while another set remained methotrexate-free to serve as control. The medium contained the normal growth medium concentration of 2 mM L-glutamine. At the time specified by the arrows 0.5 mM 5-FU was added to cultures represented by the open and closed circles and squares. All cultures were sampled at hourly intervals and acid extracted thereafter.

The symbols indicate the following: (△), GTP: - MTX, - 5-FU;(▲), HS-3: - MTX, -5-FU; (⊙), GTP: - 5-FU + MTX; (●), HS-3: - 5-FU + MTX; and (⊡), GTP: + 5-FU + MTX; (■), HS-3: + 5-FU + MTX.

promoted HS-3 accumulation. After 6 h, HS-3 in methotrexate-treated cells had increased 4-5 fold (see Fig. 2).

The purine salvage pathway mutants, YH21 and YHD13, did not consume HS-3 when purine and pyrimidine compounds were supplied. The only exceptions were adenine that is very effective, uridine that is weakly effective and the thymine analogue 5-FU. These results clearly showed that the salvage pathways were essential for the consumption of HS-3. A possible reason for adenine-induced HS-3 depletion in YHD13 is that it is leaky. Alternatively, it might have reverted and this has not been checked.

The de novo biosynthetic pathway mutant, GAT^-, provided another illuminating result. By its construction, it allowed us to study the probable role of the pyrimidine (thymidine-requiring) and purine (adenine-requiring) biosynthetic pathways, in HS-3 biosynthesis. Thymidine deficiency failed to alter the cellular level of HS-3 in this mutant but adenine deficiency did. It caused HS-3 level to increase and to decrease upon its restoration. Because GAT^- has a lesion in its 1-C transfers (10), and one such reaction occurs early in the de novo nucleotide biosynthesis pathway, we conclude that HS-3 must be produced as a consequence of a block at an early step in the de novo purine biosynthesis pathway. Indirect support of this conclusion comes from studies with methotrexate.

Effect of 5-FU on methotrexate-induced HS-3 synthesis.

Methotrexate at 1 μM stimulated HS-3 production or accumulation in 'wild type' and purine salvage mutant CHO cells (Fig. 2). Cellular HS-3 increased continuously for 6 h while GTP plateauxed after 3 h. When 5-FU was added 2 h after methotrexate feeding, HS-3 no longer increased and the cellular pool stayed constant in both types of cells (Fig. 2A and B). So, either HS-3 synthesis was halted by 5-FU or it was now in equilibrium with its consumption rate. In contrast, GTP synthesis was affected differently in the wild type and mutant cell. In the wild type, 5-FU did not alter the nature of GTP synthesis after methotrexate was added (Fig. 2A). But in the mutant, the quantity of GTP accumulating was relatively low compared to the wild type and it decreased after 2 h (Fig. 2B). Upon addition of 5-FU to the mutant after 2 h, the level of GTP continued to increase and did not drop even after 6 h. The interesting feature here is that while the blocking effect of methotrexate on de novo biosynthesis of nucleotides might not have been complete, 5-FU aided the leakage in the mutant but not in the wild type cell.

Discussion

Despite the large body of knowledge about enzymes and reactions of the purine:pyrimidine salvage pathways, their biological control remain enigmatic. Now, attempts are being made to correct this in view of the possible importance of some of these reactions in certain hereditary diseases of man, to wit, deficiency in hypoxanthine:guanine phosphoribosyl transferase activity in cells of patients with Lesch-Nyhan syndrome (12), the complex causes of gout (13) and the less common xanthuria and hereditary orotic aciduria.

A balance between de novo and salvage pathways of nucleotide biosynthesis exists but the regulatory parameters are unknown since the control of salvage pathways is not understood. What is clear is that 5-phosphoribosyl-1-pyrophosphate (PRPP), a crucial intermediate for salvage and de novo pathways, must be

involved in the regulation. The body of evidence is that intracellular PRPP is limiting in man in vivo and in cells in culture (14, 15). Therefore, factors affecting PRPP concentration most likely would affect the rate of purine biosynthesis.

The partially characterised HS-3 molecule we have uncovered in these mammalian and fungal cells may play a vital role in this regulation. To begin with, HS-3 metabolism seems to be more closely linked to purine than pyrimidine metabolism. And, the effectiveness of 5-FU in inducing HS-3 depletion may be related to the unusual uracil-end of the HS-3 molecule (see appendix). We also draw attention to the observation that cellular HS-3 level rose when de novo purine:pyrimidine nucleotide biosynthesis was arrested by withdrawing glutamine and fell when it was replenished in the appropriate cell types (table 1). HS-3 seems to be formed when an early step of de novo purine synthesis is blocked which is interesting since the level of PRPP declines upon glutamine limitation (16). HS-3 is a highly polyphosphorylated compound (see appendix). HS-3 disappears when purine bases are converted to nucleotides in cells with operational salvage pathways. ^{14}C-adenine supplied under glutamine starvation conditions was converted primarily to ADP and AMP, but, upon replenishment of glutamine, the label was detected mainly in ATP (unpublished data). The evidence is therefore strong that HS-3, in some unelucidated way, may coordinate the salvage and de novo nucleotide biosynthesis pathways in mammalian cells.

Appendix

Data to be published elsewhere will show that contrary to conclusions drawn in our early analysis (6), these HS compounds are adenine-uracil (HS-3), uracil-uracil (HS-3, HS-1) dinucleoside polyphosphate complexes. HS-3 contains a glutamyl:ADP fragment covalently linked to a pX-uracil-Y-pentaphosphate fragment where X is ribitol-like and Y, mannitol-related sugar alcohols. HS-2 and HS-1 also contain the pX-uracil-Y-polyphosphate fragment to which UDP is attached. HS-3 has 8 phosphates, HS-2, 10 phosphates and HS-1, 12 phosphates.

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