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THE EFFECTS OF TUBERCIDIN, 8-AZAADENOSINE, AND FORMYCIN ON THE SYNTHESIS AND METHYLATION OF NUCLEAR RNA IN L1210 MOUSE LEUKEMIA CELLS

HENRY J. STERN

1982

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THE EFFECTS OF TUBERCIDIN, 8-AZAADENOSINE, AND FORMYCIN ON THE SYNTHESIS AND METHYLATION OF NUCLEAR RNA IN L1210 MOUSE LEUKEMIA CELLS

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirement for the degree of Doctor of Medicine 1982.

Henry J. Stern

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ABSTRACT

Three adenosine analog antitumor agents, tubercidin, formycin, and 8-azaadenosine were investigated for their affects on the synthesis and methylation of nuclear RNA (nRNA) in L1210 mouse leukemia cells in Vitro. Tubercidin inhibited both total RNA and DNA synthesis to the greatest extent ($IC_{50} = 7 \times 10^{-6}M$) as determined by filtration assay. These effects were not potentiated by the adenosine deaminase inhibitor 2'-deoxycoformycin (dCF). Formycin and 8-azaadenosine demonstrated negligible inhibition in the absence of dCF; however, $1 \times 10^{-4}M$ formycin + dCF and $1 \times 10^{-4}M$ 8-azaadenosine + dCF each inhibited RNA synthesis by 40%, both agents showing preferential inhibition of RNA vs DNA synthesis. DEAE Sephadex-Urea chromatography of alkaline hydrolysates of nRNA double-labeled with L-[methyl-^{3}H] methionine and [^{14}C] uridine demonstrated preferential inhibition of base methylation relative to nRNA synthesis for all three drugs. This effect was not observed for 2'-O-ribose methylation. Using denaturing polyacrylamide gel electrophoresis, tubercidin was shown to preferentially inhibit methylation relative to [^{14}C] uridine incorporation in both >18S nRNA and 4S nRNA; however, formycin and 8-azaadenosine demonstrated selective preferential inhibition of methylation relative to nRNA synthesis for 4S nRNA and > 18S nRNA respectively. Both tubercidin and formycin preferentially inhibited the synthesis of 4S nRNA while 8-azaadenosine showed no consistent dose response for selective inhibition of the synthesis of either 4S nRNA or >18S nRNA. Two dimensional thin-layer chromatography of enzymatically digested 4S nRNA revealed that the observed inhibition of base methylation was associated with reduced methylation of seven of the eight methylated nucleosides commonly found in 4S nRNA, with each drug apparently inhibiting a unique spectrum of RNA methyltransferases. These differential effects of tubercidin, formycin, and 8-azaadenosine on nRNA synthesis and methylation showed no consistent relationship with their glycosyl bond lengths or torsion angles. The results of this investigation indicate that tubercidin, formycin, and 8-azaadenosine can inhibit nRNA methylation, and the hypothesis in proposed that this effect may be related to their antitumor properties via the production of defectively methylated RNA which subsequently undergoes maturation arrest and degradation.

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INTRODUCTION:

In this study the effects of tubercidin, 8-azaadenosine, and formycin on nuclear RNA synthesis and methylation in L1210 mouse leukemia cells is investigated (Stern, 1980). These compounds, structurally related to adenosine, differ only in the imidazole portion of the purine ring of the parent compound (Fig. I). Recent advances in molecular genetics have implicated that methylation of RNA is important in the processing of this informational macromolecule. That RNA methylation and neoplasia may be related comes from studies showing that certain neoplastic tissues have increased tRNA methylase capacity (Borek, 1972). By investigating the effects of these structurally similar adenosine analogs on this important biological process, it is hoped that structure activity correlates can be deduced that maybe useful in the rational design of future antineoplastic agents.

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And finally, I would like to dedicate this thesis to my mother.

-PART ONE-

CELLULAR METABOLISM AND ANTITUMOR PROPERTIES OF TUBERCIDIN, FORMYCIN, AND 8-AZAADENOSINE

TUBERCIDIN

Tubercidin, 4-amino-7-(β-D-ribofuranosyl) pyrrolo [2,3-d] pyrimidine, also known as 7 deazaadenosine is a pyrrolopyrimidine nucleoside antibiotic isolated from the broth of Streptomyces tubercidicus (Fig. I) (Anzae, 1957). The glycosyl bond length of tubercidin is 1.44 A, shorter than the glycosyl bond of adenosine which is 1.47A (Abola, 1973). Abola also found that the glycosyl torsion angle X: O-C(1')-N(9)-C(8) which describes the relative orientation of the sugar to the base of a crystal of tubercidin to be 73.0. Normally purines exist in the anti ($X= 30 \pm 45^{\circ}$) conformation and it is this conformation that is required for normal duplex formation. Although in the solid state tubercidin occurs in the anti conformation, in aqueous solution both syn ($X= 210 \pm 45^{\circ}$) and anti conformations were obtained by tubercidin and its 5' phosphate (Evans, 1975). Tubercidin is classified along with formycin and 8-azaadenosine as having a glycosyl torsion angle in the high anti range, intermediate between the classical anti and syn regions (Singh, 1977). As will be discussed below, the steric conformation of these analogs is important in determining their biochemical properties.

The in Vitro cytotoxicity of tubercidin was quickly established for NF mouse sarcoma, human epidermoid carcinoma (KB), Ehrlich ascites cells, and Earles L cells (Duvall, 1962). Unlike studies using Streptococcus faecalis, cytotoxicity in the KB system was not reversible with purine or pyrimidine nucleosides of nucleotides (Smith, 1967; Bloch, 1967). In Vivo cytotoxicity studies were also performed where toxicity to the lung,

pancreas, kidneys, liver, and gonads was observed following intravenous administration of tubercidin (Owen, 1964). It was found that of 25 tumors tested tubercidin was confirmed to be active at non toxic doses against mice with ascitic sarcoma 180, Ehrlich ascities tumor, and Jensen sarcoma. Leukemia L1210 was not affected by tubercidin treatment.

Because of these encouraging results phase I trials were undertaken. In 1970 Bisel et al selected 93 patients with a neoplastic spectrum including lung, kidney, sarcoma, colon, rectum, breast, head and neck, pancreas, melanoma, ovary, cervix, and stomach and administered intravenous tubercidin in a dose of 0.025 mg/kg/day, increasing this to 0.3 mg/kg/day in the absence of toxicity (Bisel, 1970). Such a course of treatment lasted for 10 consecutive days. Nephrotoxicity, manifested by proteinuria and azotemia, was observed in 18 cases with thrombophlebitis in 12. Leukopenia to 2900-4500 leukocytes/mm^3 occurred in 6 patients. Only three patients showed a response, 1 subjective and 2 both subjective and objective as assessed by a decrease in size of an intra-abdominal mass. All three had primarly pancreatic carcinoma of the islet cell type. There were six such patients studied.

In an effort to avoid the problem of thrombophlebitis caused by IV administered tubercidin, it was observed that 80%-98% of tubercidin is absorbed by red blood cells (Smith, 1970). Most of the absorbed tubercidin exists intracellularly in the triphosphate form. There was no decrease in tubercidin loaded RBC survival as assessed by ^{51}Cr tag experiments. When administered by rapid IV injection to dogs, 25% of

the drug appeared in the urine in 24 hours, but when red blood cells were used as a vehicle only 18% appeared in the urine in 21 days. These results were exploited by Grage who removed 300-500 ml of blood/patient, saturated it with tubercidin, and retransfused it at weekly intervals for 2 doses ranging from 200ug/kg to 1500 ug/kg in the 45 patients studied in his phase I trial (Grage, 1970). No thrombophlebitis was observed. Six cases of hematologic toxicity, 5 renal, 2 hepatic, and 1 case of severe gastrointestinal toxicity were noted and there was no correlation with increased dose and the number of patients experiencing toxicity. Of the patients studied 4 had an objective regression, 3 islet cell cancers and 1 metastatic stomach carcinoid to the cervical lymph nodes. It is of interest that in animal studies disappearance of the islets of Langerhans is consistently found in tubercidin treated animals, and it is this selective toxicity that may make tubercidin valuable in current chemotherapeutic protocols. The Central Oncology Group Protocol 7230A combines tubercidin, streptozotocin, and 5-flourouracil whereby tubercidin is mixed with 500 ml of the patient's blood and retransfused over a four hour period in a dose of 1.5 mg/kg of body weight. 5-Flourouracil at 12.5 mg/kg is given the same day and for an additional two days. After a 7-day rest period streptozotocin is added in a dose of 12.5 mg/kg in weekly doses alternating with 12.5 mg/kg of 5-flourouracil for the next six weeks. After a four week rest period the regimen is repeated if the hematologic picture is satisfactory. This protocol was valuable in treating 5 patients with extensive gastrinoma, all patients

feeling better and gaining from 3-35 pounds (Zollinger, 1976). Two of four patients with advanced islet cell carcinoma showed a dramatic initial response with the other two demonstrating prolonged survival on this protocol (Kraybill, 1976). In a four year study beginning in 1972, 105 patients with adenocarcinoma or islet cell carcinoma of the pancreas were treated with the Central Oncology Group Protocol or 5-flourouracil alone (Awrich, 1979). The results indicated that for adenocarcinoma of the pancreas there was no significant difference in survival or time to progression of disease when these two treatments were compared. Eight patients with islet cell tumors were treated with the Central Oncology Group Protocol only with none experiencing progression of the disease and three demonstrating a partial response, where a partial response was defined as a 50% reduction in the product of the diameters of the lesions.

Another example of the clinical efficacy of tubercidin is in the treatment of solitary nodular basal cell carcinoma (Burgess, 1974). Eight patients with this disorder were treated with a 0.5% ointment of tubercidin with all showing clinical disappearance of the tumor confirmed by biopsy and negative recurrence at 1 1/2 years post treatment. No signs of systemic toxicity were observed. Similar results were obtained by Klein, who in addition found higher doses of tubercidin ointment of value in the treatment of mycosis fungoides, reticulum cell sarcoma, squamous cell carcinoma, and soft tissue metastases of carcinoma of the breast (Klein, 1975).

The mechanism of the cytotoxicity of tubercidin has been investigated

on many different levels. Tubercidin has been shown to be cytotoxic to synchronized DON cells, isolated from a chinese hamster fibroblast line, in all phases of the cell cycle at low dose, while at higher doses cells at the G1-S boundry appeared more sensitive (Bhuyan, 1972). Studies on DNA, RNA, and protein synthesis using low dose tubercidin revealed marked inhibition in the synthesis of all these molecules, 84%, 86%, and 88% inhibition respectively. Simultaneous inhibition of DNA, RNA, and protein synthesis was also observed to occur in another mouse fibroblast line (Acs, 1964). This author also observed certain cytologic changes when the cells were exposed to tubercidin. These changes did not occur when the cells were pretreated with actinomycin. This is an interesting result in lieu of the data obtained by Bhuyan which suggests increased sensitivity of cells to tubercidin at a time in the cell cycle dominated by RNA transcription.

Further insight into the biochemical basis of the cytotoxicity of tubercidin comes from work with sublines of human epidermoid carcinoma (H.Ep.#2) cells with deficiencies in adenosine kinase, a subline with a combined deficiency in adenosine kinase and hypoxanthine-guanine phosphoribosyl transferase (HGPRT), and another line with a combined deficiency in adenosine kinase and adenine phosphoribosyl transferase (APRT). By determining the sensitivities of these sublines to an array of purine ribonucleoside analogs it was deduced that the three pathways illustrated in (Fig. II) could be utilized by these compounds in gaining entrance into the nucleotide pool (Bennett, 1966). Tubercidin was

shown to utilize pathway I, involving adenosine kinase, as its major anabolic pathway although pathway II, involving nucleoside phosphorylase and APRT is also used, albeit to a much lesser degree. Confirming this were studies demonstrating that tubercidin is in fact a substrate not only for adenosine kinase from H.Ep.#2 cells, being phosphorylated 4X faster than adenosine, but for adenosine kinase isolated from yeast and sarcoma 180 cells as well (Schnebli, 1967; Bloch, 1967). Data showing that mouse fibroblasts are insensitive to the aglycone of tubercidin, and that adenosine phosphorylase and APRT isolated from extracts of S. faecalis did not utilize tubercidin also suggests that pathway II is not a major anabolic pathway (Acs, 1964; Bloch, 1967). Interestingly, pathway III was shown to be the major metabolic pathway for adenosine in this cell system. It is no surprise that tubercidin is not metabolized via pathway III since this adenosine analog has been shown not to be a substrate for adenosine deaminase (ADA) purified from intestinal mucosa, human erythrocytes, or P388 murine leukemia cells (Bloch, 1967; Agarwal, 1975; Adamson, 1977). Adamson demonstrated that tubercidin inhibited the growth of P388 murine leukemia cells to the same degree, with or without 2'deoxycoformycin (dCF), a competitive inhibitor of adenosine deaminase (Ki = 2.5×10^{-12}M for human erythrocyte ADA). Characterization of the acid soluble pool of mouse fibroblasts exposed to tubercidin revealed 5-10% Tu5'MP, 8-15% Tu5'DP, and 75-85% Tu5'TP, with no guanine analogs detected (Acs, 1964). It is perhaps the inability of tubercidin to be metabolized via pathway III that accounts for its lack of conversion

to guanine nucleotides.

Numerous studies have demonstrated that tubercidin not only becomes a member of the nucleotide pool but perturbs that pool as well. Bloch demonstrated that tubercidin interfered with the formation of ATP from AMP in both the sarcoma 180 and _S. faecalis_ systems, a result confirmed in mouse leukemia EL4 cells where up to 80% of cellular ATP was lost with a concomitent build up of tubercidin triphosphate and its 3'5' cyclic analog (Bloch, 1967; Zimmerman, 1978). Using azaserine treated H.Ep.#2 cells, tubercidin was as effective as adenosine in producing a 50% reduction in formylglycinamide ribonucleotide and ribonucleoside levels (Bennett, 1964). This result was investigated further in Ehrlich ascites tumor cells in Vitro, whereby using ribonucleotide synthesis from $[^{14}C]$ adenine as a measure of the rate of 5 phosphoribosyl-1-pyrophosphate (PRPP) synthesis, tubercidin was shown to inhibit PRPP synthesis (Henderson, 1965). Therefore, while tubercidin has been shown by several studies to inhibit de novo purine biosynthesis in a variety of systems, the site of this inhibition appears to be phosphoribose pyrophosphokinase and not amidophosphoribosyl transferase as one would have guessed.

Evidence that tubercidin is capable of entering the deoxyribonucleotide pool comes from studies where tubercidin was used as a substrate and allosteric modulator for prokaryotic ribonucleotide reductase. _E. Coli_ ribonucleotide reductase, which reduces ribonucleoside diphosphates using dGTP and TTP as positive allosteric modulators, acted on tubercidin diphosphate with 40% of the activity demonstrated for ADP, with dGTP but not TTP serving as a positive allosteric modulator (Chassy, 1968).

Unlike the E. Coli enzyme, ribonucleotide reductase from Lactobacillus leichmanii utilizes ribonucleoside triphosphates with dGTP and 2'dATP as "prime effectors" (Suhadolnik, 1968). Tubercidin triphosphate reduction was 75% of ATP with dGTP acting as a better positive modulator than 2'dATP for this reduction. In addition to this, tubercidin triphosphate was found to be 50% as effective as 2'dATP as a positive allosteric modulator for CTP reduction. While 2'dATP is a negative modulator for ATP reduction, tubercidin triphosphate had a negligible effect. The results of these studies indicate that at least in prokaryotes tubercidin is a substrate for reduction to its deoxy analog, supplying precursors for incorporation into DNA, and that tubercidin can interact, albeit in a modified way with the regulatory site of this enzyme.

As has been reviewed above, tubercidin is able to enter the nucleotide pool and can be converted to deoxy as well as mono, di, and triphosphate forms. Evidence exists that these nucleotide precursors are capable of entering anabolic pathways used in the formation of nucleotide containing cofactors as in the case of nicotinamide-deaza-adenine dinucleotide isolated from tubercidin treated S. faecalis (Bloch, 1967). Tubercidin incorporation into DNA and RNA has been established in bacteria, mouse fibroblasts, mouse L5178y cells, and the single stranded RNA Mengo virus (Bloch, 1967; Acs, 1964; Seibert, 1978). Seibert demonstrated that with L5178y cells treated with a dose of tubercidin of 0.05ug/ml 21% is incorporated into acid insoluble material with 15% of the tubercidin incorporated distributed into DNA and 84%

into total RNA. Acs, using tubercidin at a dose of 1 ug/ml to treat mouse fibroblast L cells, found a total incorporation of tubercidin into acid insoluble material of only 5% with a distribution of tubercidin into RNA 2.7X that of DNA (Acs, 1964). It appears then that incorporation of tubercidin into acid insoluble material varies with the cell system studied, and that tubercidin appears to accumulate in RNA to a greater extent than DNA.

Several studies have demonstrated that tubercidin interferes with RNA transcription and processing. This evidence will be presented later.

FORMYCIN

Formycin A or 7-amino-3-(β-D-ribofuranosyl)-pyrazolo [4,3-d] pyrimidine was originally isolated from culture filtrates of Nocardia Interforma (Hori, 1964). As can be seen from (Fig. I), the structure of formycin (formycin will be used to refer to formycin A) is similar to adenosine except that C8 and N9 have been interchanged causing an increase in the length of this CC glycosyl bond to 1.55 A (Ward and Reich, 1968). The glycosyl torsion angle is 109.5, much larger than the angle for tubercidin, but like tubercidin exists in a high anti configuration (Prusiner, 1973). The absence of the C8 hydrogen, which in adenosine interacts with the furanose ring to decrease rotation, combined with this increase in glycosyl bond length, allows for freedom of rotation in this adenosine analog not observed in the parent compound. Formycin has been shown to be a substrate for P388 murine leukemia adenosine deaminase, being deaminated to the inosine analog formycin B (Fig. III), with a Km and Vmax larger than adenosine (Adamson, 1977). Adenosine deaminase isolated from calf intestines has been shown to accept substrates only in the anti conformation and the increased Km observed for formycin may be explained by the conformation of this analog which is intermediate between syn and anti (Ogilvie, 1971). Presumably the freedom of rotation around the glycosyl bond allows formycin to adopt the anti conformation required, and it is subsequently deaminated. The results of Crabtree challenge this hypothesis though (Crabtree, 1979). Using N-methylformycins

sterically restricted in either the syn or anti conformation as substrates for human erythrocyte adenosine deaminase, he demonstrated that this enzyme is insensitive to the syn anti conformation variable. Agarwal suggests that the increase in Vmax observed for formycin relative to adenosine may be accounted for by the N7, N8 diazo bond which would make C6 more electropositive and therefore favor nucleophilic displacement (Agarwal, 1975). As mentioned earlier, tubercidin was shown by this author not to be a substrate for adenosine deaminase. Formycin and 8-azaadenosine are substrates; however, and it is suggested that the imidazole portion of the purine ring of adenine, in particular the N7 position, appears to be an important structural variable in the activity of this enzyme.

The product of the adenosine deaminase reaction, formycin B, can be considered a detoxified product of formycin A. While formycin A was shown to have an ID50 of 1.0 uM for L1210 leukemia cells, formycin B had an ID50 of 75 uM in this same system (Crabtree, 1979). By adding 2'deoxycoformycin the percent growth inhibition of P388 murine leukemia cells exposed to formycin A was increased from 5% to 71% in the presence of this adenosine deaminase inhibitor (Adamson, 1977). These results were largely confirmed in viral systems as well where formycin A, but not formycin B, were shown to inhibit the multiplication of vesicular stomatitis and vaccinia viruses; however, for influenza A virus both agents were equally effective in inhibiting multiplication (Takeuchi, 1966; Giziewicz, 1975).

In addition to its cytotoxic effects on P388 and L1210 cells, formycin has been shown to be cytotoxic to Yoshida rat sarcoma cells and Ehrlich carcinoma cells, cytotoxicity being potentiated in both systems by coformycin, another adenosine deaminase inhibitor (Ki human erythrocyte ADA 1 X 10^{-11}M Agarwal, 1977). Umazawa demonstrated that daily intraperitoneal injections of formycin into mice with ascitic Ehrlich ascites carcinoma prolonged the survival of the mice 3 fold, and that the same increase in survival could be obtained with a four fold lower dose of formycin if coformycin were administered concomitantly (Umezawa, 1967). This author further demonstrated that formycin B was not cytotoxic to Ehrlich ascites carcinoma cells and that no phosphorylated derivitives of this analog could be detected intracellularly, unlike formycin A, which was cytotoxic and was found to exist intracellularly in mono, di, and triphosphate forms. Further insight into the metabolism of formycin was obtained by Sheen who injected mice with formycin and found 50% of the injected dose in the urine as oxoformycin (Fig. III), the xanthosine analog of formycin B (Sheen, 1969). It was demonstrated that the conversion of formycin B to oxoformycin was catalized by hepatic aldehyde oxidase and that both formycin B and formycin A are competitive inhibitors of xanthine oxidase. These results are surprising in lieu of the fact that inosine, the structural analog of formycin B, does not interact with hepatic aldehyde oxidase, nor does adenosine or inosine interact with xanthine oxidase. Oxoformycin has been shown to be ineffective as a cytotoxic agent against Yoshida sarcoma cells

(Kunimoto, 1968).

In synchronized cultures of HeLa S3 cells, high concentration formycin (10 ug/ml) inhibited DNA synthesis while low dose formycin (0.1 ug/ml) inhibited protein synthesis by 25% (Kunimoto, 1967). Kunimoto also demonstrated that the cells were sensitive to inhibition of cell division by low dose formycin at a stage of early DNA synthesis. As mentioned above, DON fibroblasts appeared to be more sensitive to the cytotoxic effects of tubercidin at the GIS boundry suggesting perhaps a similarity of cell cycle sensitivity to these two agents.

In discrepancy with these results is the work of Giziewicz who showed a 95% reduction in both RNA and DNA synthesis of primary rabbit kidney cells exposed to 200 ug/ml formycin, with an 80-90% reduction in RNA and DNA synthesis respectively when these cells were exposed to 40 ug/ml formycin (Giziewicz, 1975). It appears that lower dose formycin inhibited RNA synthesis less, and the lack of inhibition of RNA synthesis observed by Kunimoto may be explained by an insufficiently broad dose response curve.

As is the case with tubercidin, the biochemical basis for the cytotoxicity of formycin has been investigated by numerous authors. Formycin was shown not to be incorporated into the nucleotide pool of human erythrocytes, where tubercidin was incorporated and converted to the triphosphate form (Parks, 1973). This result has not been repeated in other systems however. Mouse leukemia EL4 cells exposed to formycin were shown to accumulate formycin triphosphate with a dose and time

dependent decrease in cellular ATP, though less of a decrease than was observed with tubercidin (Zimmerman, 1978). The cyclic analog of formycin was also demonstrated. Using intraperitoneal injections of formycin into mice carrying a line of ascitic Ehrlich ascites carcinoma cells lacking adenosine kinase, formycin was shown to be ineffective against the kinase deficient line while inhibiting the parent line by 95% at the same dose (Caldwell, 1969).

That formycin is indeed a substrate for adenosine kinase was established using adenosine kinase purified from H.Ep. #2 cells, where formycin was found to be phosphorylated at the same rate as adenosine (Schnebli, 1967). Further insight into the biochemistry of formycin was obtained by Henderson who measured N-formylglycinamide ribonucleotide levels in Ehrlich ascites tumor cells exposed to azaserine and formycin (Henderson, 1967). In these experiments he demonstrated a 10 fold decrease in PRPP synthesis in the parent line as compared to an adenosine kinase deficient line. This inhibition of PRPP synthesis, which presumably occurs at the level of phosphoribose pyrophosphokinase, was also observed in tubercidin treated cells (Henderson, 1965). In addition to inhibiting de novo purine biosynthesis, formycin has been shown to decrease nucleotide synthesis from hypoxanthine and guanine, but not from adenine, in Ehrlich ascites tumor cells (Henderson, 1967). As this author suggests, formycin maybe inhibiting HGPRT directly.

While formycin has not been demonstrated in Vitro or in Vivo to be incorporated into DNA, it has been shown to be a substrate for ribonucleotide

reductase from Lactobacillus leichmannii where in the absence of dGTP formycin simply binds to the active site, while in the presence of dGTP it is reduced (Ward, 1969; Brinkley, 1978). With respect to CTP reduction, formycin triphosphate was observed to be ineffective as a positive allosteric modulator; however, deoxyformycin triphosphate appeared to be as effective as dATP and deoxytubercidin triphosphate (Suhadolnik, 1968; Brinkley, 1978).

It appears then that evidence exists that formycin is theoretically capable, at least in prokaryotes, to enter the deoxyribonucleotide pool; however, unlike tubercidin it has an absolute requirement for the binding of dGTP to the allosteric site of ribonucleotide reductase for this to occur. Formycin also appears to interact with the allosteric site of this enzyme differently than tubercidin, in that only deoxyformycin triphosphate is capable of functioning as an allosteric modulator while both tubercidin triphosphate and deoxytubercidin triphosphate are effectors.

As can be seen, both formycin and tubercidin can substitute for adenosine as a substrate in numerous enzyme systems. This is not universally true however. Unlike tubercidin, which was found to substitute for adenosine in nicotinamide adenine dinucleotide (NAD), formycin has not been shown to be incorporated into NAD nor was it a substrate for purified NAD synthase (Ward, 1969). This author further demonstrated that formycin triphosphate could substitute for ATP as an energy source for amino acid esterification to tRNA by aminoacyl-tRNA synthase, yielding rates of esterification 50%-75% of control depending on the

amino acid substrate. On the other hand, tubercidin triphosphate was shown to be incapable of serving as an energy source for this enzyme (Uretsky, 1968).

At the level of cellular metabolism concerned with RNA translation, both tubercidin triphosphate and formycin triphosphate are utilized by tRNA CCA pyrophosphorylase, substituting for adenosine in the 3' terminal CCA group characteristic of tRNA. The tRNAs thus formed are capable of accepting amino acids, except for tRNA^{Phe}CCTu, which shows a 20 fold decrease in aminoacetylation with Phe. In addition, these molecules appear to vary in their ability to substitute for naturally occurring tRNA in cell free systems (Baksht, 1975). With respect to messenger function, both tubercidin and formycin substitute for adenosine with fidelity; however, polyF does not code for polylysine while polyTu does so. Studies on the stimulation of binding of aminoacyl tRNA to ribosomes with formycin or tubercidin containing ribonucleotide polymers has demonstrated normal to decrease binding for formycin, but normal binding for tubercidin (Ikehara, 1965; 1969). In summary, the results of these studies seem to suggest that both tubercidin and formycin can substitute for adenosine in processes concerned with messenger translation. This is an interesting result in lieu of the data presented earlier that both compounds inhibited protein synthesis in cytotoxicity studies. That the inhibition of protein synthesis results from a derangement at the level of RNA transcription is a possibility; however, in mouse fibroblasts a simultaneous inhibition of DNA, RNA, and protein synthesis

was observed when these cells were exposed to tubercidin (Acs, 1964). The rapidity with which tubercidin inhibits protein synthesis would suggest that tubercidin in some way directly interferes with RNA translation.

At the level of RNA transcription these analogs appear to behave differently when used as substrates by RNA polymerase. While tubercidin triphosphate is utilized as a substrate by Mengo virus induced RNA polymerase and QB replicase, formycin triphosphate is not (Kapuler, 1969). That formycin triphosphate is a substrate for _E. Coli_ RNA polymerase has been demonstrated using numerous templates where the rate of polymerization varied from 23%-34% depending on the template (Ward, 1969). Slightly higher rates of polymerization of formycin triphosphate were observed by Ikehara, 47%-68%, using _E. Coli_ RNA polymerase and various templates (Ikehara, 1968). Acs reported that tubercidin triphosphate could be used in homopolymer formation; however, Nishimura, using _E. Coli_ RNA polymerase, demonstrated that the rate of incorporation varied with the template and that no homopolymer formation could be detected (Acs, 1964; Nishimura, 1966). Formycin triphosphate was not used in homopolymer synthesis by _E. Coli_ RNA polymerase (Ward, 1969). The results of these studies are significant in that they indicate that with respect to these sterically different adenosine analogs, incorporation into RNA is dependent not only on Watson Crick type base pairing, but on the steric conformation of the neighboring base as well.

In rifampicin challenge experiments DNA dependent RNA polymerase

from Azotobactor vinelandii initiated chains with tubercidin triphosphate at a faster rate than ATP (Kumar, 1977). When formycin triphosphate was used as a substrate for chain initiation; however, the t1/2 for this reaction was 17X larger than the value for ATP. In phosphodiester bond formation formycin triphosphate was far more efficient than tubercidin triphosphate in single step addition while in polymerization it performed only slightly better. Darlix also observed a decrease in chain initiation with normal rates of elongation when formycin triphosphate was used as a substrate for E. Coli RNA polymerase (Darlix, 1971). In addition to this, he observed that formycin prevented the release of newly synthesized RNA chains from the transcription complex.

It is interesting that these structurally similar adenosine analogs would affect different aspects of RNA transcription differently, and one can only speculate as to the steric variable that restricts formycin in its ability to initiate RNA chains. Kumar concluded that only analogs in the anti conformation are suitable substrates for the initiation of RNA chains, thereby explaining why tubercidin with a glycosyl torsion angle of 73.0 is a better initiator than formycin with an angle of 109.5 (Abola, 1973; Prusiner, 1973). The situation appears to be more complicated than this; however, in that Evans demonstrated that in aqueous solution tubercidin, like formycin, exists in syn anti equilibrium (Evans, 1975). Whatever the explanation formycin, because of its poor ability to initiate RNA chains and its retarding effect on RNA chain release, can be expected to disrupt RNA transcription to a significant

extent. The effects of formycin on RNA processing will be discussed below.

8-AZAADENOSINE

9-β-D-ribofuranosyl-8-aza-adenine is the third adenosine analog used in this investigation and unlike the other two compounds, which are naturally occurring nucleoside antibiotics, 8-azaadenosine is chemically synthesized (Fig. I). Since its first reported synthesis in 1958, not much data has been accumulated on the mechanism of action of this triazolo adenosine analog (Davoll, 1958). It belongs to the general category of 8-azapurine nucleosides such as formycin and 8-azaguanosine, and like formycin and tubercidin exists in an intermediate anti syn conformation with a glycosyl torsion angle of 103.6, very similar to the angle of formycin (Singh, 1974; 1977). Unlike formycin which has an increased glycosyl bond length, the glycosyl bond of 8-azaadenosine is 0.02 A shorter than adenosine. The replacement of C8 with nitrogen not only distorts the angles of the imidazole moiety of the parent compound, and provides an additional basic site, but makes C6 more electropositive as in the case of formycin. This variable, as well as the syn anti conformation of 8-azaadenosine, is used to explain the higher Km and Vmax when compared to adenosine using purified human erythrocyte adenosine deaminase (Agarwal, 1975). Since tubercidin is not a substrate, these authors concluded that N7 is important in substrate binding; however, in 8-azaadenosine N7 is not protonated and therefore unavailable for hydrogen bonding (Singh, 1974). Taken together this would imply that N7 may be important in functioning in activities other than hydrogen bonding.

Similar results were obtained with adenosine deaminase isolated from P388 murine leukemia cells, and in one experiment percent growth inhibition was increased from 13% to 57% in the presence of dCF (Adamson, 1977).

In addition to adenosine deaminase, 8-azaadenosine has been shown to be a substrate for purified adenosine kinase from H.Ep. #2 cells, being phosphorylated 1.9X faster than adenosine (Schnebli, 1967). Using these same cells in culture [H^3] 8-azaadenosine was found to be converted to mono, di, and triphosphate forms (Allan, 1972). In human erythrocytes 8-azaadenosine was found to be incorporated as the monophosphate only (Parks, 1973).

Antitumor activity of 8-azaadenosine has been demonstrated using L1210 and P388 murine leukemia cells where mice carrying these tumors intraperitoneally had a percent increase in life span of 42% and 70% respectively when treated with 8-azaadenosine (Montgomery, 1975). The mechanism of this cytotoxicity is difficult to elucidate in that unlike tubercidin and formycin, which are not metabolized to other potent antineoplastic agents, 8-azaadenosine is. As early as 1957 it was recognized that 8-azaadenine inhibited the growth of tobacco mosaic virus and _E. Coli_ (Smith, 1957). Smith suggested that this inhibition was correlated with the incorporation of 8-azaguanine into RNA by showing that no 8-azaadenine could be detected in the alkaline hydrolizates of the RNA. In H.Ep. #2 cells 8-azaadenosine was found to be 20X more toxic than its aglycone, implying low conversion of this substrate by APRT (Montgomery, 1975). A subline of these cells deficient in adenosine kinase, and another

subline with a combined deficiency in adenosine kinase and APRT, were both shown to have the same degree of resistance to 8-azaadenosine implying that this substrate is being metabolized via pathway III in (Fig. II) to account for its cytotoxic effects (Bennett, 1966). Further elucidation of this mechanism was obtained using a line of cells with a combined deficiency of adenosine kinase and HGPRT where the degree of resistance rose to approximately 30X the resistance demonstrated in the two sublines mentioned previously (Montgomery, 1975). Since this adenosine kinase HGPRT deficient subline was similarly resistant to 8-azainosine, these authors concluded that the mechanism of cytotoxicity of 8-azaadenosine resided in its ability to be metabolized to 8-azainosine. A similar proposal had been made 3 years earlier by Allen who demonstrated that [^{3}H] 8-azaadenosine was converted by the parent line of H.Ep. #2 cells to 8-azaAMP, 8-azaADP, 8-azaATP, as well as small amounts of 8-azaIMP (Allan, 1972). When a subline deficient in adenosine kinase and APRT was incubated with [^{3}H] 8-azaadenosine, only a small quantity of 8-azaIMP could be detected, yet 8-azaadenosine was equally cytotoxic to both cell lines. The authors concluded that it was 8-azaIMP that was the cytotoxic end product of 8-azaadenosine metabolism. Further insight into these cytotoxic metabolites was obtained by Bennett who demonstrated that 8-azainosine increased the life span of mice carrying intraperitoneal L1210 leukemia cells by 40%-50%, and that this compound was equally effective against a line lacking HGPRT and therefore resistant to 8-azahypoxanthine (Bennett, 1973). Similar results have

also been obtained by Bennett using H.Ep. #2 cells in culture and confirmed by Montgomery (Montgomery, 1975). These data imply the existence of an inosine kinase as an alternative route of inosine metabolism. When H.Ep. #2 cells were treated with 8-azainosine the degree of resistance was observed to increase 20 to 40 fold in a subline with a combined deficiency in adenosine kinase and HGPRT, as compared to a subline lacking HGPRT alone (Montgomery, 1975). These authors concluded that either 8-azainosine is a substrate for adenosine kinase or that a closely linked inosine kinase was subsequently lost when the adenosine kinase deficient line was selected for. Similar results were obtained by Bennett to explain the cytotoxicity of 7 deazainosine in these same sublines (Bennett, 1966). Subsequent studies demonstrated that while [^{14}C] 8-azainosine was converted almost exclusively to 8-azaguanine nucleotides by H.Ep. #2 cells, [^{14}C] 8-azaadenosine was converted to the nucleotides of adenine and guanine equally (Bennett, 1976). Similar results are obtained when incorporation into polynucleotides (DNA + RNA) were examined. 8-azaadenosine treated cells showed equal incorporation of adenine and guanine nucleotides, while 8-azainosine treated cells showed exclusive incorporation as 8-azaguanine. In a H.Ep. #2 subline lacking adenosine kinase and APRT, both 8-azaadenosine and 8-azainosine treated cells demonstrated approximately 90% incorporation into polynucleotides as 8-azaguanine. The authors concluded that 8-azaIMP is a substrate for IMP dehydrogenase and GMP synthase, but it is not used by the enzymes in the anabolic pathway from IMP to AMP. In addition to this; however, one can conclude that the 50% incorporation of

adenine observed in the parent line treated with 8-azaadenosine is due to metabolism by adenosine kinase or APRT. Considering the low toxicity of 8-azaadenine mentioned earlier it can be concluded that pathway I in (Fig. II), which utilizes adenosine kinase, shares equally with pathway III the metabolism of 8-azaadenosine. When a subline of the H.Ep. #2 cells resistant to 8-azainosine but sensitive to 8-azaadenosine was treated with 8-azaadenosine, 90% incorporation as adenine was observed in the polynucleotide fraction. This line was shown not to be deficient in adenosine kinase or HGPRT; however, it did show an approximately 50% reduction in the ability to convert 8-azainosine to 8-azahypoxanthine (Bennett, 1973). This data becomes interpretable if one assumes a concomitant deficiency in inosine kinase, thereby leaving only the adenosine kinase pathway accessible for the metabolism of the 8-azaadenosine, and no pathway accessible for the metabolism of 8-azainosine in this 8-azainosine deficient line.

In summary, the cytotoxicity of 8-azainosine may be linked to its incorporation into DNA and RNA as 8-azaguanine. While Bennett did not further define the incorporation into DNA vs RNA, the majority of 8-azaguanine is most likely incorporated into RNA since negligible amounts of 8-azaguanine has been noted to be incorporated into DNA in other systems (Parks, 1974). The basis of the cytotoxicity of 8-azaadenosine may also be linked to its incorporation into DNA and RNA as observed in the 8-azainosine resistant subline. In the parent line; however, one cannot conclude that cytotoxicity is attributable to

8-azaadenosine alone since extensive conversion to 8-azaguanine metabolites also occurs. It is interesting in this regard that Adamson observed an increase in cytotoxicity of 8-azaadenosine to P388 leukemia cells when these cells were also treated with dCF. dCF, by inhibiting adenosine deaminase, would be expected to block conversion of 8-azaadenosine to 8-azaguanosine.

Like tubercidin and formycin, 8-azaadenosine has been shown to be a feedback inhibitor of de novo purine biosynthesis in azaserine treated H.Ep. #2 cells (Bennett, 1964). It is not known; however, if this inhibition occurs at the level of amidophosphoribosyl transferase or phosphoribose pyrophosphokinase, as was observed for tubercidin and formycin (Henderson, 1965; 1967).

In the preceding sections the cellular metabolism of the three antitumor agents tubercidin, formycin, and 8-azaadenosine has been reviewed. While these compounds are dissimilar only in that they vary in the imidazole portion of the purine ring of the parent compound adenosine, these subtle steric differences are responsible for their biochemical properties and ultimately their cytotoxic potential. In the next section evidence will be presented that these compounds disrupt RNA processing, and that their ability to interfere with the methylation of RNA may be a mechanism by which this occurs.

-PART TWO-

METHYLATION AND EUCARYOTIC RNA PROCESSING

In this section the role of methylation in eucaryotic RNA processing will be briefly reviewed.

HETEROGENEOUS NUCLEAR AND MESSENGER RNA

The process begins with RNA transcription and in eucaryotes three RNA polymerases have been identified based on their different sensitivities to the inhibitor α-amanitin, isolated from the poisonous mushroom *Amanita phalloides*. In animal cells RNA polymerase II is the most sensitive and it is this polymerase that generates heterogeneous nuclear RNA (hnRNA). As has been reviewed by Lewin, the majority of radioactive label incorporated into hnRNA turns over in the nucleus, implying extensive intranuclear degradation of this molecule (Lewin, 1980). Heterogeneous nuclear RNA has an average length of 8000-10,000 bases and presumably is the precursor of mRNA, which has an average length of 2000-2100 nucleotides, as suggested by pulse chase experiments whereby the 3' terminal poly A and 5' methyl caps of hnRNA have been shown to be conserved in mRNA. The function of these terminal modifications remains obscure but it has been suggested that they may play some role in message selection from the large pool of hnRNA precursors. Only 70% of mRNA from HeLa cells is poly adenylated, specifically histone mRNA has been shown to lack a poly A tail, arguing against the necessity of a poly A tail for message transport. 5' methyl caps have been shown to be more ubiquitous with only certain viruses such as polio, encephalomyocarditis, tobacco necrosis, satellite tobacco necrosis virus, and yeast killer particle RNA lacking caps (Muthukrishnan, 1978). Transfer RNA (tRNA) and ribosomal RNA (rRNA) also lack caps

but are transported to the cytoplasm, again demonstrating that terminal modification is not required for message transport.

As can be seen in (Fig. IV), the 5' cap consists of a 7-methyl-guanosine linked by a 5'-5' pyrophosphate bridge to a 2'-O-ribose methylated base. This structure is designated as cap 1, and while all the caps of hnRNA are of this type, only 65% of the caps on messenger RNA are cap 1 (Cori, 1975). Twenty percent of mRNA caps contain adenosine in the second position which is further methylated at N^6 to generate dimethyl (2'-O; N^6) adenosine. The remaining 15% of 5' terminal caps of mRNA are designated as cap 2, where the third base from the 5' end is additionally methylated at the 2'-O-ribose position.

The putative mechanism for the generation of these caps is summarized in (Fig. V) where the capping reaction is believed to occur after the initiation of transcription; however, in cytoplasmic polyhedrosis virus evidence has been obtained suggesting that cap formation may occur as early as the initiation step (Furuichi, 1979). Similar evidence exists for reovirus and possibly vaccinia virus as well (Shatkin, 1976). Following initiation with a purine nucleoside, guanine is added via guanyl transferase and is subsequently methylated to 7-methylguanine by 7-methyltransferase with S-adenosylmethionine (SAM) as a methyl donor. Next, 2'-O-methyl transferase utilizes SAM to methylate the ribose of the second base, which for mRNA 64% of bases analyzed is a purine. Since transcription is initiated with a purine, this implies that at least 36% of caps are generated from internally cleaved precursors.

For those caps having N^6, 2'-0-dimethyladenosine, the base methylation reaction has been shown to follow the 2'-0-ribose modification (Keith, 1978). This enzyme is specific for the cap structure and is not believed to be involved in the generation of internal N^6-methyl adenosine residues. As appears to be the case for transmethylation reactions in general, these methyl transferases are inhibited by S-adenosylhomocysteine (SAH), the product of SAM coupled transmethylations (Coward, 1973).

As reviewed by Shatkin, the 5' terminal cap appears to protect RNA from degradation thereby influencing message turnover (Shatkin, 1979). In addition to this, several studies have indicated that the cap structure is important in the efficient translation of message. Vaccinia virus mRNA with an unmethylated 5' terminal guanosine was shown to have decreased binding to ribosomes, as well as poor ability to stimulate protein synthesis, in wheat germ or rabbit reticulocyte lysate systems (Muthukrishnan, 1976). Restoration of ribosome binding capacity and message function were observed when guanine 7-methyltransferase was used to remethylate the terminal base. These authors also demonstrated that 2'-0-methylation has significantly less influence on ribosome binding under the in Vitro conditions used. Similar results were obtained from studies on the binding of cap modified vesicular stomatitis virus mRNA, reovirus mRNA, and capped synthetic ribopoymers to rabbit reticulocyte or wheat germ ribosomes (Muthukrishnan, 1976). By using an RNA (2'-0-methyladenosine-N^6)-methyltransferase isolated from HeLa cells to generate a dimethyladenosine cap 1 structure on vaccinia virus

mRNA, Muthukrishnan showed that this base modification did not increase the ribosome binding capacity of this mRNA as compared to unmodified cap 1 containing vaccinia mRNA. From these studies it can be concluded that base methylation of the terminal guanosine in the 5' terminal caps of RNA has profound effects on ribosome binding, where 2'-O-ribose methylation and additional base methylations contribute minimally to this reaction, at least under the in Vitro conditions examined.

In addition to the methylated nucleosides found in the 5' terminal caps of both hnRNA and mRNA, both of these molecules contain internal m^6A and small amounts of m^5C, except for HeLa histone and globin mRNA (Moss, 1977; Perry, 1975). m^6A appears to be distributed in the trinucleotides $GA^{me}C$ (70%) and $AA^{me}C$ (30%) both in hnRNA and mRNA from HeLa and L cells (Wei, 1977; Schibler, 1977). This similar distribution of N^6 methyl adenosine in both hnRNA and mRNA, combined with the observation that an increase to 1.0 m^6A/1000 bases of mRNA from 0.4 m^6A/1000 bases of hnRNA occurs, is taken as evidence for the precursor product relationship of hnRNA to mRNA, and that internal methylations may be involved in this process.

RIBOSOMAL RNA

Another example of conserved internally methylated bases in RNA maturation is ribosomal RNA processing (Fig. VI). In eucaryotes RNA polymerase I transcribes a 45s precursor rRNA molecule from clusters of tandemly repeated ribosomal genes in the nucleolus. This precursor molecule contains the sequences of 18s rRNA, which is present in the 40s ribosomal subunit, as well as the 5.8s and 28s rRNA sequences which are found in the 60s ribosomal subunit. The 5s rRNA of the 60s ribosomal subunit is transcribed at a separate locus by RNA polymerase III.

As has been extensively reviewed, HeLA cell 18s rRNA contains 45 methyl groups, 7 of which are base methylations. 28s rRNA has 68 methylated groups, 5 of which are base methylations, and 5.8s rRNA contains 1 and possibly 2 methyl groups (Burdon, 1971; Maden, 1979; Lewis, 1980). As can be seen the majority, approximately 90% of the 110-115 total methylated nucleotides, are methylated at the 2' hydroxyl position of the ribose. These sugar methylations are believed to occur rapidly after transcription while the base modifications which include 1-methyladenine, N^6-methyladenine, N^6-dimethyladenine, 1-methylguanine, N^7-methylguanine, N^2-dimethylguanine, 7-methylguanine, 3-methylcytosine, and 3-methyluracil, are believed to occur later. 5s rRNA is unmethylated.

Using [^{3}H] methionine and [^{32}P] phosphate to label the RNA of HeLa cells infected with polio virus, which causes an accumulation of ribosomal RNA intermediates, Weinberg elucidated the maturation pathway in (Fig. VI) (Weinberg, 1970). The 45s precursor is entirely methylated except for

two adjacent dimethyladenosines, which later appear in the 18s species. This precursor gives rise to a 41s intermediate, which is then cleaved to a 32s and 20s fragment. The 20s species is further processed to 18s rRNA, while the 32s fragment goes on to 28s and 5.8 s rRNA. By showing that the ratio of $[^{3}H]$ methyl groups to $[^{32}P]$ labeled nucleotides increased during the maturation process, Weinberg was able to demonstrate that the sequences lost were unmethylated and that the methyl groups on the original 45s precursor transcript were preserved quantitatively. As it turns out, roughly 50% of the 13,000 nucleotides in the original 45s precursor are the unmethylated G-C rich transcribed spacers, later to be discarded in the maturation process.

That methylation is important in this maturation scheme is suggested by the results of methionine deprivation experiments in HeLa cells (Vaughan, 1967). HeLa cells deprived of methionine but not valine continue to transcribe the 45s precursor and process it to the 32s intermediate, despite the fact that there are 5X and 3X fewer 2'-O-ribose methylations respectively. No further processing was observed in the methionine deprived cells, and in fact, further intermediates were shown to be degraded. By adding back $[^{14}C]$ methionine, after treating the cells with actinomycin D to block further transcription, Vaughan showed that label entered the 45s and 32s rRNA species and could be detected in ribosomal RNA isolated from the cytoplasm. He concluded that methylating enzymes recognize specific sites independent of the length of the molecule or its stage in maturation.

It is true that all the methylated sites possess different primary sequences, but as Maden points out, this would require separate methylating enzymes for each sequence leading to a highly complex scheme (Maden, 1974). He suggests that the functional role of 2'-0-methylation is to protect against nucleolytic degradation, and therefore only exposed sequences would be methylated at the 2' hydroxyl position by enzymes recognizing this conformational feature. Using nuclease S_1, which hydrolyzes single stranded regions in the nucleic acids, this conformational hypothesis was tested for HeLa cell 18s rRNA (Khan, 1978). The results indicated that it was the four base methylated sites, modified late in maturation, that were accessible to nuclease S_1, and therefore exposed, with less than 50% of the sites carrying 2'-0-ribose methyl groups in exposed positions. The unexposed methylated sites were presumably modified early in the maturation process by methylating enzymes that apparently recognize primary sequence, later to be burried by secondary and tertiary interactions.

As can be seen, ribosomal RNA maturation is a process that conserves methylated nucleotides. Whether these methylated bases provide specific recognition sites for the ribonucleases involved in this process, or simply protect the various intermediates from degradation remains to be elucidated.

TRANSFER RNA

Transfer RNA is the transcription product of RNA polymerase III, and while being only 73-93 nucleotides in length, it is the most highly modified nucleic acid in cells (for reviews see Burdon, 1971; Nau, 1976; and Smith, 1976). The familiar cloverleaf structure of tRNA is depicted in (Fig. VII) with the amino acid arm, the anticodon arm, extra arm if present, and the Tψ (pseudouridine) C arms representing the leaves of the cloverleaf. On the average there are 6 to 7 methylated nucleotides/tRNA in eucaryotes and unlike rRNA, ribose methylation accounts for only 10%-20% of the total methylation. The species of methylated bases identified in the tRNA of HeLa and mouse cells include 1-methyladenine, 1-methylguanine, N^2-methylguanine, N^2, N^2-dimethylguanine, 7-methylguanine, 5-methylcytosine, and 5-methyluracil with 1-methylhypoxanthine, 3-methylcytosine, and 3-methyluracil occurring in minor amounts.

Unlike ribosomal RNA maturation, the role of methylated nucleotides in tRNA processing is obscure. Using [^{14}C] uridine to label the small RNA of HeLa cells, radioactivity was found to be incorporated into material migrating between 5s ribosomal and 4s tRNA (Bernhardt, 1969). In actinomycin D block pulse chase experiments, label in this putative 4.5s pre-tRNA was chased into tRNA and further experiments demonstrated that both pre-tRNA and tRNA incorporated [^{14}C] methylmethionine. A possible role for methylation in this maturation process was suggested by methionine starvation experiments where HeLa cells deprived of methionine were shown to have a decreased rate of conversion of pre-tRNA

to tRNA, as well as a decreased rate of synthesis of the precursor. Addition of methionine, but not vlaine, restored to normal levels the rate of conversion of the precursor tRNA to its mature form. Bernhardt also noted that the undermethylated tRNA produced accumulated in the cytoplasm, and he concluded that complete methylation is not essential for tRNA maturation.

Since this early observation, 4.5s pre-tRNA has been observed in numerous systems including chinese hamster ovary and Ehrlich ascites tumor cells (Choe, 1972). Analogous to the ribosomal RNA precursor, pre-tRNA from yeast has been shown to contain intervening sequences which are subsequently removed in the maturation process (Knapp, 1978). The location of the maturation events is also a subject of debate; however, in experiments with HeLa cells a crude cytoplasmic extract was shown to convert pre-tRNA to tRNA suggesting that the cytoplasm is the locus of tRNA processing (Bernhardt-Mowshowitz, 1970).

Further insight into the relationship between methylation and tRNA processing was obtained by Munns (1975). Using human carcinoma KB cells in labeled methionine pulse chase experiments, Munns was able to determine specific methylation events as a function of tRNA maturation by varying the time of the initiation of the pulse relative to the actinomycin D block. The results indicated that late stage methylations included 2'-O-methylribose, N^2-methylguanine, and 3-methylcytosine. Intermediate stage modifications included 1-methyladenine, 5-methylcytosine, and 5-methyluracil while the early methylated nucleotides

were 1-methylguanine, 7-methylguanine, and N^2, N^2-dimethylguanine. As was pointed out, the late modifications appear restricted to the anticodon loop and the dihydrouridine arm, while the intermediate stage methylated nucleotides are found in the TψC loop and arm. Early methylated nucleotides appear to reside around the junctions of the major arms (Fig. VII). Munns concluded that selected regions of tRNA become methylated at specific times during the maturation of pre-tRNA.

The enzymes responsible for the generation of these methylated nucleotides, the S-adenosylmethionine tRNA methyltransferases, are a group of "base specific, species specific, and organ specific" enzymes that in eucaryotes specifically require S-adenosylmethionine (SAM) as a methyl group donor (Kerr, 1972). They are highly sensitive to competitive inhibition by the product of the transmethylation reaction, S-adenosylhomocysteine (SAH), with Ki values ranging from 6.3×10^{-6}M to 2×10^{-5}M for various enzymes (Nau, 1976). The cellular location of these enzymes is not known with certainty. Cell fractionation techniques using Krebbs II mouse ascites cells located these enzymes predominately in the cytoplasm (Burdon, 1967). Similar results were obtained in rat liver cells using in Vivo labeling techniques (Muranatsu, 1968). As Burdon points out; however, aqueous media was used in the cell fractionation procedures and the possibility therefore exists that the methylating enzymes were extracted from the nuclei during isolation. The majority of tRNA methylase activity has been reported to be associated with nuclei when non-aqueous media was used (Kahle, 1971).

While numerous studies have demonstrated that the tRNA methyltransferases are a group of tRNA specific, base and base position specific enzymes, Nau suggests that they can be divided into two general categories. The "common methylases" are responsible for the modification of those bases at sites that are found to be methylated on many different tRNA species such as 2'-0-methylguanosine #19, 7-methylguanosine #55, 5-methyl uridine #70, and in eucaryotes additionally 1-methylguanosine #9, N^2-methylguanosine #10, N^2, N^2-dimethylguanosine #29, 5-methylcytidine #64 and #65, and 1-methyladenosine #74. "Specific methylases" would be responsible for the generation of the pattern of methylation distinctive of each tRNA species.

The biologic role of methylated bases in tRNA remains obscure. Although the experiments by Bernhardt mentioned previously would seem to indicate that complete methylation is not critical for tRNA maturation, certain key methylations may in fact be important in this process. The data obtained by Munns showing that specific methylations occur in a time ordered sequence of pre-tRNA maturation supports this point of view. That methylated nucleotides are important in establishing and maintaining the unique tertiary structure of tRNA has been proposed, and in support of this is the observation that the majority of methylated nucleotides occur in non-base paired regions. Only N^2, N^2-dimethylguanosine and 5-methylcytosine, which do not prevent G-C hydrogen bonding, are found in helical regions. To date however, a specific functional role of methylated nucleotides in tRNA has not been unambiguously assigned.

RNA PROCESSING IN DRUG TREATED CELLS

Earlier in this section the maturation of various species of RNA in eucaryotes was reviewed, and experimental evidence suggesting that methylated nucleotides are important in RNA processing was examined. Tubercidin and formycin have been demonstrated by several authors to disrupt RNA processing. Polyacrylamide agarose gel electrophoresis of total cellular RNA isolated from Novikoff hepatoma cells exposed to 1 ug/ml tubercidin for 2 hours showed approximately 80% inhibition of incorporation of [^{3}H] uridine into 32s rRNA, with greater than 95% inhibition of incorporation of label into 28s and 18s rRNA as compared to control (Weiss, 1974). Incorporation of [^{3}H] uridine into 45s and 38s rRNA was the same in control and treated cells. (Novikoff hepatoma 38s rRNA is similar to the 41s rRNA precursor of HeLa cells.) L5178Y mouse tumor cells treated with 0.05 ug/ml tubericdin for 6 hours were shown by similar analytic techniques to contain a "high molecular weight precursor RNA" not isolated from control cells (Seibert, 1978). Although this material was not characterized, it seems possible that it may be high molecular weight precursor rRNA. These authors further demonstrated that tubercidin treated cells contained decreased amounts of 4s and 5s RNA as compared to untreated cells.

Interference with ribosomal RNA processing has also been observed with formycin. HeLa cells treated with 25 ug/ml of formycin for 10 minutes followed by a 90 minute pulse of [^{3}H] uridine exhibited delayed processing of 45s rRNA to 32s rRNA (Abelson, 1973). With a 30 minute

[^{3}H] uridine pulse followed by actinomycin D to block transcription and allow processing of pre-tRNA to the mature 4s form, these authors also demonstrated that cells treated with formycin showed significant inhibition of cytoplasmic 4s and 5s RNA at a concentration of drug not affecting the synthesis of hnRNA or 45s ribosomal precursor. This differential inhibition of low molecular weight RNA synthesis was also observed in formycin treated posterior silk glands of the silk worm Bombyx mori (Majima, 1977). Using pulse chase experiments with [^{3}H] uridine, these authors made the important observation that the conversion of 4.5s pre-tRNA to tRNA was significantly inhibited in formycin treated glands, while the rate of incorporation of label into pre-tRNA and the disappearance of pre-tRNA was the same as control. Glands exposed to [^{14}C] formycin were shown to incorporate formycin into total RNA as well as pre-tRNA, but only negligibly into 4s RNA. The authors concluded that formycin containing precursor tRNA was degraded by a scavenger mechanism, and in a subsequent study when formycin containing pre-tRNA was incubated with a crude ribosomal wash from the silk glands, no 4s RNA was observed to accumulate (Tsutsumi, 1978). 4.5s precursor tRNA isolated from control cells was converted to 4s material by this extract.

In summary, the above experiments suggest that formycin inhibits ribosomal as well as tRNA processing from their respective precursors. Tubercidin likewise inhibits ribosomal RNA maturation, and although tubercidin triphosphate was shown by Seibert to inhibit DNA dependent RNA polymerases I, II, and III isolated from mouse liver (Seibert, 1978), the studies by Majima and Tsutsumi cast doubt on the hypothesis that

inhibition of RNA transcription can be used solely to explain the decreased amounts of cytoplasmic 4s RNA observed in the tubercidin treated cells.

As mentioned in the previous section, HeLa cells deprived of methionine were shown to contain undermethylated precursor ribosomal RNAs that did not complete maturation due to degradation of further intermediates (Vaughan, 1967). Similarly, methionine starved HeLa cells demonstrated a decreased rate of conversion of pre-tRNA to tRNA (Bernhardt, 1969). Although Weiss, Majima, and Tsutsumi proposed that the mechanism of the interference in RNA maturation in tubercidin and formycin treated cells was due to incorporation of these analogs into RNA leading to molecules with anomalous properties, when considered in light of the methionine deprivation experiments another mechanism becomes plausible whereby these drugs interfere with RNA methylation, and it is the generation of improperly methylated precursors that accounts for the inhibition of RNA processing observed. Evidence does in fact exist that these analogs, as well as 8-azaadenine, can inhibit various tRNA methylases in Vitro, albeit at very high concentrations.

Using submethylated tRNA and enzyme extracts from E. Coli, lumole/ml 8-azaadenine was shown to inhibit tRNA methylation by 10% (Moore, 1970). Tubercidin, in concentrations ranging from approximately 300 to 400 ug/ml inhibited tRNA methylation in crude enzyme extracts of calf liver, E. Coli, and calf spleen by 19%, 24%, and 30% respectively (Wainfan, 1967). More specifically, tubercidin was shown to inhibit

guanine tRNA methyltransferases from E. Coli, and when uracil tRNA methyltransferase was examined approximately 1 mM tubercidin inhibited this enzyme by 44%, while at a similar concentration formycin inhibited this enzyme by only 2% (Wainfan, 1975).

It can be seen that the evidence that these adenosine analogs can interfere with RNA methylation is restricted to in Vitro studies, mostly with procaryotic tRNA methyltransferases and at very high concentrations of inhibitor. The present study was designed to investigate the effects of tubercidin, formycin, and 8-azaadenosine on the synthesis and methylation of nuclear RNA in L1210 mouse leukemia cells. Because these adenosine analogs are dissimilar only in that they vary in the imidazole portion of the purine ring of the parent compound, it is hoped that specific structure activity correlates can be deduced.

-PART THREE-

THE EFFECTS OF TUBERCIDIN, 8-AZAADENOSINE, AND FORMYCIN ON THE SYNTHESIS AND METHYLATION OF NUCLEAR RNA IN L1210 MOUSE LEUKEMIA CELLS

ABBREVIATIONS:

nRNA, nuclear RNA; SDS, sodium dodecylsulfate; TCA, trichloroacetic acid; dCF, 2'-deoxycoformycin [(R)-3-(2-deoxy-β-D-erythropentofuranosyl)-3,6,7,8-tetrahydroimidazo [5,4-d] [1,3] diazepin-8-ol], m^1A, 1-methyl-adenosine; m^3C, 3-methylcytidine; m^5C, 5-methylcytidine; m^1G, 1-methylguanosine; m^2G, N^2-methylguanosine; m_2^2G, N^2, N^2-dimethylguanosine; m^7G, 7-methylguanosine; m^5U, 5-methyluridine; IC_{50}, median inhibitory concentration.

MATERIALS AND METHODS:

Materials: Tubercidin, 8-azaadenosine, formycin, and dCF were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. [5-^{3}H-methyl] Thymidine (20Ci/mmole), L-[methyl-^{3}H] methionine (80Ci/mmole), and [U-^{14}C] uridine (464 mCi/mmole) were purchased from the New England Nuclear Corporation, Boston, Massachusetts. m^1A, m^3C, m^5C, M^1G, M^2G, M_2^2G, m^7G, and m^5U were obtained from P-L Biochemicals, Milwaukee, Wisconsin.

Animals: L1210 cells were inoculated, i.p., into BALB/c X DBA/2 F_1 mice at an inoculum of 10^5 cells/0.1 ml Hanks' balanced salt solution. Cells were harvested 6 days after inoculation and were further diluted with incubation medium.

Incubations: Incubations of L1210 cells were carried out at 37° in a shaking water bath at 100 r.p.m. and consisted of: (1) 25 ml of L-methionine-free RPMI 1630 medium, 5 X 10^7 cells, 200uCi [^{3}H] methionine (80 Ci/mmole) and 5 uCi [^{14}C] uridine (464 mC /mmole) or (2) 5 ml of RPMI 1630 medium, 1 X 10^7 cells, 1uCi [^{14}C] uridine (464mCi/mmole) and

5 uCi [^{3}H] thymidine (20 Ci/mmole). Cells were preincubated with 1uM dCF for 15 minutes before further incubation for 30 minutes with either tubercidin, 8-azaadenosine, or formycin. The period of labeling was 1 hour.

Total RNA and DNA Synthesis: Incorporation of [^{14}C] uridine and [^{3}H] thymidine into total RNA and DNA respectively was measured by cooling the incubation flasks on ice for 15 minutes and adding ice-cold TCA to a final concentration of 10% (w/v). Precipitates were collected on glass fiber filter discs, washed 3X with 5% TCA and 2X with 95% ethanol. Discs were dried and counted in 10 ml Aquasol in a Searle Mark III liquid scintillation system.

RNA Extraction: After incubation, cells were centrifuged at 400g for 20 minutes at 4° C, washed once with cold RPMI 1630 medium, and recentrifuged at 400 g for an additional 10 minutes at 4° C. Nuclei were prepared from the cell pellet by resuspension in 10 ml of 10mM magnesium acetate swelling medium (pH 5.1) for 15 minutes at 4° C. This swelling medium was previously found to markedly inhibit the activity of intracellular RNase, thereby minimizing the hydrolysis of nRNA before extraction. The samples were then adjusted to room temperature by stirring for 10 minutes at room temperature, followed by the addition of 0.15 ml of Triton X-100 for an additional 5 minutes. Nuclei were collected by centrifugation at 2000 g for 10 minutes at 4° C and resuspended in 5 ml of 0.34 M sucrose, 5mM magnesium chloride which was layered on a cushion of 5 ml of 0.88 M sucrose, 5 mM magnesium chloride, and centrifuged at 2000g for 10 minutes at 4°C. nRNA was extracted from the pelleted nuclei by vortexing vigorously for 2 minutes with 3 ml of 0.1% SDS: 0.14M NaCl: 0.025M sodium acetate

(pH 5.1) and 3 ml of phenol mixture (phenol-m-cresol-water (7:2:2), v/v) containing 0.1% 8-hydroxyquinoline. The emulsion was clarified by centrifugation at 12,000g for 10 minutes, and the upper aqueous phase was removed and precipitated with 3 vol. of 2% potassium acetate in 95% ethanol at -20°C overnight.

DEAE Sephadex-Urea Chromatography: precipitated RNA was collected by centrifugation at 10,000g for 20 minutes at -10° C. The precipitate was washed with cold 95% ethanol and recentrifuged at 10,000g for 10 minutes at -10° C. The pelleted RNA was hydrolyzed at 37° C overnight in 0.5 ml of 0.3 N potassium hydroxide. Phenolphthalein was added as an indicator and the pH adjusted with perchloric acid till slightly basic. The samples were allowed to stand on ice for 20 minutes followed by centrifugation at 2000g for 10 minutes at 4° C to remove the potassium perchlorate precipitate. The supernatant was diluted to 10 ml in buffer: 20mM Tris HCl (pH 7.6), 7 M urea and absorbed onto a 0.9 cm X 15 cm column of DEAE sephadex equilibrated in buffer. The column was washed with 10 ml of buffer and eluted with a 200 ml linear gradient of 0.1 M NaCl to 0.7 M NaCl 2 ml fractions were collected, mixed with 15 ml of Hydroflour, and counted in a Searle Mark III liquid scintillation spectrometer.

Electrophoresis: nRNA was resolved by electrophoresis in cylindrical polyacrylamide gels (0.4 X 7 cm) containing: 8% (w/v) acrylamide, 0.32% (w/v) diallyltartardiamide, 6 M urea, 0.1% (w/v) SDS, 0.2% (w/v) ammonium persulfate, 0.04% (v/v) N, N, N', N'-tetramethylenediamine,

0.4 M Tris-acetic acid (pH 7.2), 0.02 M sodium acetate, and 0.002 M EDTA. RNA samples containing one A_{260} unit were mixed with sample buffer to give a final concentration of: 0.04 M Tris-acetic acid (pH 7.2), 0.02 M sodium acetate, 0.002 M EDTA, 0.02% bromphenol blue and 20% (w/v) sucrose (RNase-free). Gels were electrophoresed at 4mA/gel at 4° C. Gels were sectioned into 2 mm slices, dissolved in 2% (w/v) periodic acid at 37° C for 15 minutes, mixed with 10 ml Aquasol, and counted in a Searle Mark II liquid scintillation spectrometer.

Thin-Layer Chromatography: 4S RNA was isolated by polyacrylamide gel electrophoresis. 4S RNA was sliced from the appropriate section of the gel and sectioned into 2 mm slices. RNA was extracted from four gel slices with 1 ml of RNA extraction buffer (0.1% SDS: 0.014 M NaCl: 0.025 M sodium acetate, pH 5.1) by continuous vortexing at room temperature for one hour. The gel was removed by centrifugation at 16,000g for 2 minutes in an Eppendorf centrifuge, and the RNA was precipitated at -20° C for 2 hours. Enzymatic digestion was carried out for 18 hours at 37° C in 20 ul of 0.05 M Tris-HCl (pH 8.0): 5mM $MgCl_2$ containing: 6 ug RNase A, 5 ug calf intestine alkaline phosphatase (1000 units/mg) and 10 ug snake venom phosphodiesterase. Samples were freeze dried and reconstituted with 20 ul of a standard mixture containing the eight methylated nucleosides at a concentration of 0.4 mg/ml. An aliquot of 5 ul was spotted on 0.25 mm silica gel plates containing fluorescent indicator (EM Laboratories, Elmsford, New York), and the eight methylated nucleosides were separated by two-dimensional thin-layer chromatography with acetonitrile-concentrated

NH_4OH (4:1, v/v) for the first dimension and acetonitrile-2 N HCOOH (10:1, v/v) for the second dimension. Silica gel was recovered from the area corresponding to the appropriate standard, mixed with 10 ml of Aquasol, and counted in a Searle Mark III liquid scintillation spectrometer.

RESULTS

Initial experiments were designed to assess the effects of tubercidin, 8-azaadenosine, and formycin on total RNA and DNA synthesis in the presence and absence of 1×10^{-6}M dCF (Fig. VIII). It is apparent that inhibition of both RNA and DNA synthesis by 8-azaadenosine and formycin are potentiated in the presence of the adenosine deaminase inhibitor, while tubercidin inhibited the synthesis of both of these macromolecules to the same extent irrespective of pretreatment with 1 uM dCF. Lower doses of tubercidin inhibited DNA synthesis more than RNA synthesis but as the dose was increased the synthesis of both RNA and DNA became inhibited to the same degree with an IC_{50} of approximately 7×10^{-6}M for both macromolecules. In the presence of dCF, 1×10^{-5}M and 1×10^{-4}M 8-azaadenosine inhibited RNA synthesis by 40% but DNA synthesis by only 10%-15%. Similarly, 1×10^{-4}M and 1×10^{-3}M formycin plus dCF inhibited RNA synthesis by 40%-80% and DNA synthesis by 30%-40%. 1×10^{-6}M dCF, which was used throughout all the experiments, did not affect RNA or DNA synthesis.

To examine the effects of these analogs on RNA synthesis and methylation, nRNA was double-labeled with L-[methyl-^{3}H] methionine and [^{14}C] uridine, and the alkaline hydrolysates chromatographed on DEAE Sephadex (Table 1, Fig. IX). All three drugs preferentially inhibited methylation relative to [^{14}C] uridine incorporation only in the mononucleotide fraction (-2 charge) representing methylated bases. This differential inhibition was not evident in either the dinucleotide fraction

(-3 charge) representing 2'-O-methylation, or in the tetranucleotide fraction (-5 charge) representing 5' cap structures. Alkaline hydrolysates from cells treated with dCF plus formycin demonstrated an additional peak containing 1.7% of the total incorporated [^{3}H] radioactivity which eluted before the mononucleotide (-2) peak (Fig. IX panel D). This material was not characterized further. 1×10^{-6}M dCF did not affect the synthesis or methylation of nRNA.

Because of the observation that tubercidin, dCF plus 8-azaadenosine, and dCF plus formycin preferentially inhibited base methylation, nRNA was next fractionated using electrophoresis in denaturing polyacrylamide gels to determine if these effects were largely restricted to 4S nRNA which is rich in methylated bases (Table 2, Fig. X). At 4×10^{-6}M tubercidin, methylation was preferentially inhibited relative to [^{14}C] uridine incorporation for both >18S nRNA and 4S nRNA. 1×10^{-5}M 8-azaadenosine plus dCF; however, preferentially inhibited methylation for only >18S nRNA, while 1×10^{-4} formycin plus dCF preferentially inhibited the methylation of only 4S nRNA. By increasing the concentration of these drugs 10 fold, the preferential inhibition of methylation relative to synthesis was no longer observed, with the exception of the effect dCF plus 8-azaadenosine on >18S nRNA. By considering only the [^{14}C] uridine incorporation data, it is apparent that 4×10^{-6}M tubercidin inhibited the synthesis of >18S nRNA and 4S nRNA to approximately the same extent, but when the dose was increased to 4×10^{-5}M the synthesis of 4S nRNA was more severely inhibited (Table 2). dCF plus 1×10^{-5}M 8-azaadenosine appeared to inhibit [^{14}C] uridine

incorporation into 4S nRNA more than >18S nRNA, but at a 10 fold higher dose no differential inhibition of the synthesis of these two classes of nRNA could be detected. In the case of dCF plus 1 X 10^{-4}M formycin; however, 4S nRNA synthesis was inhibited to a greater degree than >18S nRNA synthesis, and this differential inhibition of low molecular weight nRNA was also observed at the 10 fold higher dose.

To determine if these drugs inhibited the same, or a different spectrum of base methylating enzymes, methylated nucleosides isolated from 4S nRNA were resolved by two-dimensional thin-layer chromatography (Table 3). It appears that the degree of methylation of m^1A was relatively unaffected by any of these drugs. 4 X 10^{-6}M tubercidin appeared to markedly affect the methylation of most of the other nucleosides rather broadly, with the possible exception of m^5C and m^5U. dCF plus 1 X 10^{-5}M 8-azaadenosine seemed to have a more specific effect, inhibiting the methylation of m^3C more severely than the other nucleosides. dCF plus 1 X 10^{-4}M formycin; however, appeared not to markedly inhibit the methylation of m^3C, as did tubercidin and dCF plus 8-azaadenosine, but did severely inhibit the methylation of m^5C, which the other two drugs appeared not to do. Other than this apparently specific effect, dCF plus 1 X 10^{-4}M formycin markedly inhibited the methylation of most of the nucleosides with the exception of m^1A and m^3C mentioned earlier, and possibly m^2G and m^5U as well.

DISCUSSION

In examining the affects of tubercidin, 8-azaadenosine, and formycin on total RNA and DNA synthesis as measured by incorporation of labeled uridine and thymidine respectively into TCA precipitable material, it was determined that the effects of both formycin and 8-azaadenosine were potentiated by dCF, while tubercidin was insensitive to the action of this adenosine deaminase inhibitor. These results are consistent with the high level of adenosine deaminase in L1210 cells and are in agreement with the data obtained by Adamson who demonstrated that 8-azaadenosine and formycin, but not tubercidin, inhibited the growth of P388 mouse leukemia cells to a greater extent in the presence of dCF (Lepage, 1969; Adamson, 1977). Also in support of this data are the numerous in Vitro studies demonstrating that tubercidin is not a substrate for purified adenosine deaminase, while 8-azaadenosine and formycin are. As discussed under Cellular Metabolism, 8-azaadenosine is extensively metabolized to 8-azaguanine nucleotides by what appears to be pathway III in (Fig. II). dCF, however, would block pathway III and it can be assumed, in the absence of unknown purine pathways, that the effects of 8-azaadenosine on dCF pretreated cells are in fact due to 8-azaadenosine, and not some other metabolic product.

From the gel electrophoresis study it appears that lower dose tubercidin inhibited the synthesis of >18S nRNA and 4S nRNA to the same extent, while at the higher dose 4S nRNA was more specifically inhibited. The "high molecular precursor RNA" reported by Seibert to be isolated

only from tubercidin treated, but not from control, L5178y mouse tumor cells was not observed in this study (Fig. X panel B) (Seibert, 1975). Specific inhibition of 4S RNA synthesis was also observed with formycin,* both at high and low doses, while 8-azaadenosine probably did not specifically inhibit the synthesis of one species of nRNA vs the other since no consistent dose effect relationship could be ascertained.

The observation that formycin specifically inhibits the synthesis of low molecular weight RNA has also been made in HeLa cells and in the posterior silk gland of the silk worm Bombyx mori (Abelson, 1973; Majima, 1977). The results obtained by Majima and Tsutsumi (1978) suggesting that in formycin treated silk glands precursor tRNA did not process to the mature 4S form, and that it was degraded by a scavenger mechanism, forces one to interpret with caution RNA synthesis data based on [^{14}C] uridine incorporation into TCA precipitable material. Although no data exists that tubercidin or 8-azaadenosine treated cells produce unstable RNA, it is not unreasonable to assume in absence of data to the contrary, that these drugs may also cause the synthesis of unstable RNA, later to be degraded by some scavenger pathway.

Weiss, Majima, and Tsutusmi proposed that the mechanism of interference in RNA processing in tubercidin and formycin treated cells was due to incorporation of these analogs into RNA leading to molecules

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*In this section, when results of this study are referred to, formycin = formycin + dCF data, and 8-azaadenosine = 8-azaadenosine + dCF data.

with anomalous properties. In this study tubercidin, formycin, and 8-azaadenosine all were shown to preferentially inhibit base methylation relative to synthesis of nuclear RNA, supporting the hypothesis that defective methylation may be the common denominator for the interference in RNA processing observed in drug treated cells. It is interesting to note in this regard that formycin, which was reported to interfere with the processing of pre-tRNA to tRNA (Majima, 1977), preferentially inhibited the methylation of 4S nRNA at a dose of 1×10^{-4}M (Table 2).

If degradation of unstable RNA is occurring, and especially if it is undermethylated precursors that are being degraded, then the ratio of [^{3}H methyl] methionine to [^{14}C] uridine incorporation will be artificially increased, and one would interpret this as there being a less specific effect on the inhibition of methylation vs synthesis. For instance, the base methylation data (-2 charge) in (Table 1; Fig. IX, panels B,C,D) would demonstrate an even more pronounced specific inhibition of methylation compared to synthesis in the absence of these conjectural degradative pathways. Since 2'-O-ribose methylation is believed to protect RNA from nucleolytic degradation, it is possible that in the drug treated cells the lack of a specific inhibition of ribose methylation relative to [^{14}C] uridine incorporation in the dinucleotide fraction (-3 charge) may be an artifact due to degradation of molecules lacking appropriate 2'-O-ribose methylations.

As mentioned previously, the lower dose of formycin used preferentially inhibited the methylation of 4S nRNA relative to > 18S nRNA (Table 2).

8-azaadenosine had the opposite effect, specifically inhibiting the methylation of > 18S nRNA, while tubercidin appeared to inhibit the methylation of both of these species to the same extent. This specificity of inhibition was largely abolished at the higher concentrations of the drugs used, probably because of the severe inhibition of RNA synthesis at the higher dose, except for 8-azaadenosine treated cells which maintained a comparable rate of RNA synthesis at the two concentrations investigated.

Examination of the methylated nucleosides contained in 4S nRNA suggested that each drug inhibited its own spectrum of RNA methylating enzymes (Table 3). While the 4S nRNA species isolated from the 8% denaturing polyacrylamide gels was not characterized further, it most probably represents precursor tRNA as well as some small nuclear RNA species and possibly mature tRNA, since these gels are not capable of resolving 4S from 4.5S RNA species. It is apparent that neither tubercidin, 8-azaadenosine, nor formycin inhibited m^1A to any significant degree. If the assumption is made that L1210 cells process precursor tRNA to tRNA by the same scheme of time and position ordered base methylations worked out for KB cells by Munns (1975), then by comparing the data in (Table 3) to this scheme certain generalizations with respect to inhibition of groups of enzymes may become apparent. Both tubercidin and formycin more severely inhibited the methylation of those nucleosides clustered around the junctions of the major arms, and m^7G which is found only in the extra arm, those methylations occurring early

in pre-tRNA processing (Fig. VII). These methylated bases are also related by the fact they are all guanine nucleosides. In addition to this effect, tubercidin also inhibited to a greater extent those bases clustered in the anticodon loop and dihydrouridine arm, the late stage methylations. Formycin showed relative sparing of this region, but demonstrated severe inhibition of m^5C, which neither tubercidin nor 8-azaadenosine appeared to do. It is interesting to note that 8-azaadenosine showed the most specific effect on the inhibition of base methylation, where m^3C was inhibited the most of the 8 methylated nucleosides examined.

With respect to structure activity correlation, the drugs in (Table 3) are arranged in order of increasing glycosyl bond length and increasing glycosyl torsion angle from top to bottom. Increasing the glycosyl bond length and torsion angle is theoretically associated with increasing freedom of rotation around the glycosyl bond, allowing the compound to assume either the syn or anti conformation. When the data are examined in this way no pattern of inhibition of methylated nucleosides becomes apparent. Formycin, unique amongst the compounds studied with its C-C glycosyl bond, seemed to inhibit the methylation of m^5C more and m^3C less than the other analogs. 8-azaadenosine, the only triazolo nucleoside investigated, seemed to affect m^3C more specifically than the other drugs.

When the synthesis and methylation of $>$ 18S nRNA and 4S nRNA are examined with respect to the structural variables of glycosyl bond

length and torsion angle, again no pattern of inhibition of synthesis or methylation becomes apparent. 8-azaadenosine did not show specificity in inhibition of nRNA synthesis as did formycin and higher dose tubercidin, but did show specificity in the ability to inhibit the methylation of >18S nRNA.

Several studies have shown that tubercidin, 8-azaadenosine, and formycin are all extensively metabolized in cells and the question arises as to whether the inhibition of methylation of nRNA observed in this study is due to the direct effects of these drugs, or to some metabolic product. While this question was not addressed experimentally in this study, examination of (Fig. XI), which summarizes the eucaryotic cellular metabolism of S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), brings several speculations to mind. The first possibility, alluded to earlier, is that these drugs directly inhibit the SAM dependent RNA methyltransferases. Alternatively, they or their nucleotide derivatives could inhibit methionine adenosyltransferase, and by lowering the intracellular concentration of SAM, inhibit more severely those methyltransferases with a higher Km for SAM. If the triphosphate derivatives of these drugs are suitable substrates for methionine adenosyltransferase, then the formation of an analog containing counterpart of SAM may lead to a molecule capable of inhibiting the methyltransferase enzyme. Lastly, by progressing further along the pathway shown in (Fig. XI), the homocysteinyl derivative of the analog would be formed, and it is possible that it is the homocysteinyl derivative of tubercidin, 8-azaadenosine, and formycin that is responsible for inhibiting the RNA methyltransferases.

Using a crude preparation of tRNA methyltransferases isolated from rat liver, SAH and S-tubercidinylhomocysteine (STH) were shown to have almost identicle Ki values, 25 uM and 20 uM respectively (Coward, 1974). When more purified methyltransferases from the same source were examined, 8-azaadenosylhomocysteine was shown to be a better inhibitor of adenine-1 methyltransferase than STH or SAH, but STH and SAH were the superior inhibitors of N^2-guanine-I and guanine-1 methyltransferase respectively (Leboy, 1978). In another methyltransferase reaction, STH inhibited Newcastle Disease Virion mRNA (guanine-7-)-Methyltransferase with a Ki 36% of SAH, while 8-azaadenosylhomocysteine inhibited this methyltransferase with a Ki 8.5X larger than SAH (Pugh, 1977).

Several authors have demonstrated that STH is capable of inhibiting in Vivo RNA methylation. STH was shown to inhibit base methylation, as well as 2'-0-ribose methylation, as determined by the appearance of cap 0 and decreased amounts of cap 2 structures in cytoplasmic poly A containing RNA isolated from treated Novikoff hepatoma cells (Kaehler, 1977). Phytohemagglutinin-stimulated rat lymphocytes exposed to STH demonstrated a 49% inhibition of methylation of tRNA, and when the methylated nucleosides of tRNA isolated from STH treated Novikoff hepatoma cells were examined, m_2^2G and m^7G showed 90% and 83% inhibition of methylation respectively, while m^1A and m^5U were uninhibited (Chang, 1975; Coward, 1979). This is an interesting result when compared to the data obtained in this study where tubercidin inhibited the methylation of m_2^2G and m^7G to a greater extent than m^1A and m^5U.

The question of whether tubercidin of S-tubercidinylhomocysteine is responsible for inhibiting methylation is most directly addressed by the research of Kaehler who studied the methylation of poly A containing RNA isolated from Novikoff hepatoma cells treated with either homocysteine, adenosylhomocysteine, tubercidin, or tubercidinylhomocysteine (Kaehler, 1979). By examining the ratio of the incorporation of [^{3}H-methyl] methionine to [^{14}C] uridine, Kaehler showed that STH had specifically inhibited methylation relative to [^{14}C] uridine incorporation, while tubercidin did not. In this study, 4 X 10^{-5}M tubercidin was shown to abolish the preferential inhibition of methylation of $>$ 18S nRNA observed at 4 X 10^{-6}M, and it is possible that the lack of specific inhibition of methylation observed by Kaehler using tubercidin at a dose of 2.5 X 10^{-4}M was due to too high a dose of tubercidin. In reference to 2'-0-ribose methylation, Kaehler further demonstrated that cap 0 structures were isolated only from STH, but not tubercidin treated cells; however, tubercidin treated cells demonstrated 13X and 20X more cap 2 structures than control or STH treated cells respectively. In this study tubercidin appeared not to specifically inhibit 2'-0-ribose methylation (Table 1, -3 charge; Fig. IX, panel B), but as discussed earlier possible degradation of RNA lacking appropriate 2'-0-ribose methylation may have obscured any specificity in the inhibition of 2'-0-ribose methylation by tubercidin. The DEAE sephadex-urea chromatography technique used in this study was of insufficient resolving power to separate the different species of 5' caps and so no conclusions can be made in reference to them.

In summary, other studies have shown that tubercidin and formycin

interfere with RNA processing and it has been suggested that incorporation of these drugs into RNA is responsible for this effect. In this investigation tubercidin and formycin, as well as 8-azaadenosine, were shown to inhibit the methylation of nuclear RNA in L1210 mouse leukemia cells by a mechanism that remains unknown at present. This inhibition of methylation of nRNA suggests an alternative hypothesis whereby the production of defectively methylated RNA is responsible for the reported interference in RNA maturation by these drugs. It would be interesting to investigate other agents that interfere with RNA processing to see if they too have any effect on the methylation of RNA. Alternatively tubercidin, 8-azaadenosine, and formycin, because of their ability to inhibit RNA methylation, may prove useful in probing the role of methylation in the fascinating process of RNA maturation.

-PART FOUR-

FIGURES AND TABLES

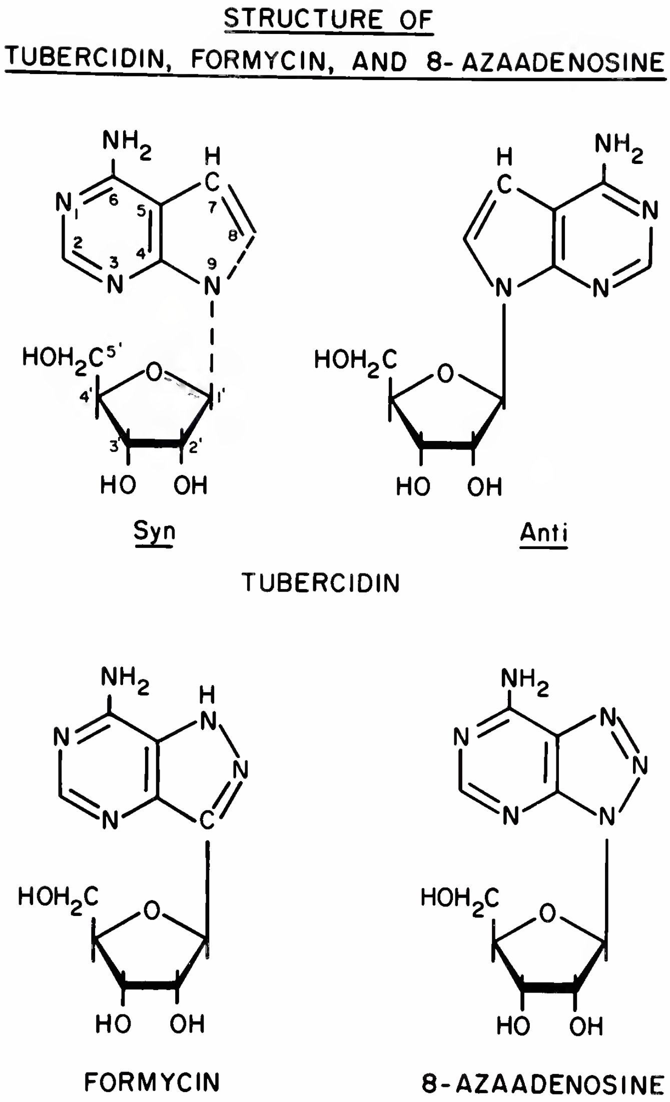
STRUCTURE OF
TUBERCIDIN, FORMYCIN, AND 8-AZAADENOSINE
Syn
Anti
TUBERCIDIN
FORMYCIN
8-AZAADENOSINE

Figure I

Dashed bonds in tubercidin illustrate angles used in the calculation of the glycosyl torsion angle.

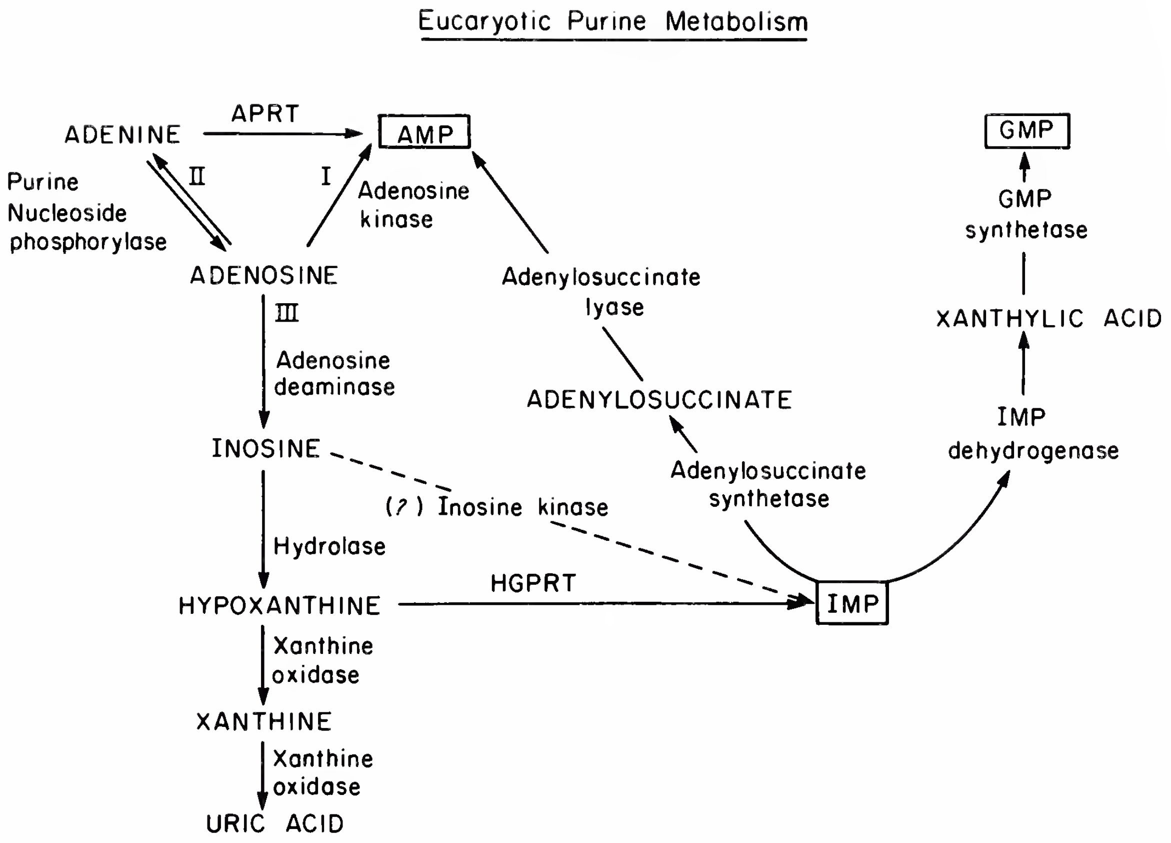
Eucaryotic Purine Metabolism
ADENINE
APRT
AMP
GMP
Purine
Nucleoside
phosphorylase
II
I
Adenosine
kinase
GMP
synthetase
ADENOSINE
Adenylosuccinate
lyase
III
XANTHYLIC ACID
Adenosine
deaminase
ADENYLOSUCCINATE
IMP
dehydrogenase
INOSINE
Adenylosuccinate
synthetase
(?) Inosine kinase
Hydrolase
HGPRT
HYPOXANTHINE
IMP
Xanthine
oxidase
XANTHINE
Xanthine
oxidase
URIC ACID

Figure II

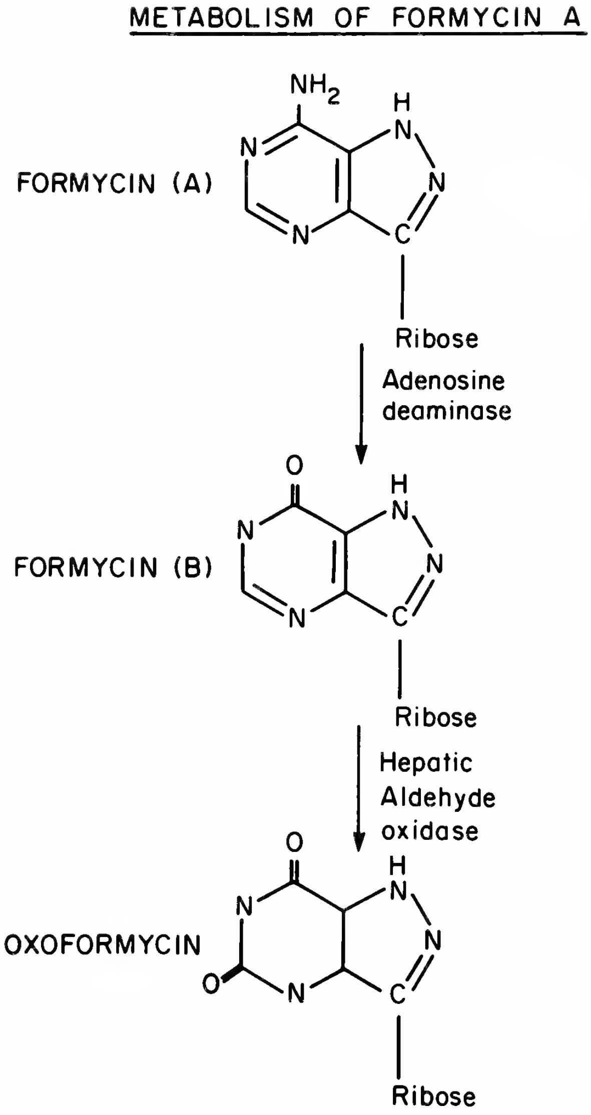
METABOLISM OF FORMYCIN A
FORMYCIN (A)
NH2
H
N
N
N
N
C
Ribose
Adenosine deaminase
FORMYCIN (B)
O
H
N
N
N
N
C
Ribose
Hepatic Aldehyde oxidase
OXOFORMYCIN
O
H
N
N
N
N
O
C
Ribose

Figure III

Structure of the 5'Terminal Cap

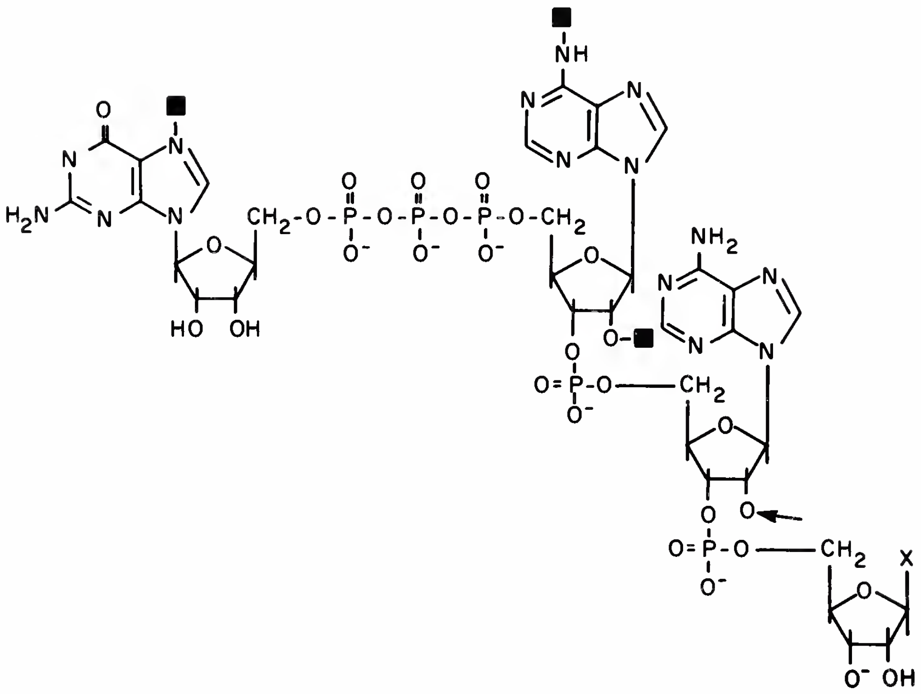
O
N
H2N
N
N
N
HO
OH
CH2-O-P-O-P-O-P-O-CH2
O
O
O
O-
O-
O-
NH
N
N
N
N
O
O-
O=P-O
O-
CH2
NH2
N
N
N
N
O
O
O
O=P-O
O-
CH2
X
O
O- OH

Figure IV

Illustrated is the structure of $^{7Me}G(5')ppp(5')A^{2'OMe}_{N_6Me}pApX$, a Cap 1 structure. Filled squares are methyl groups. Arrow indicates position of additional methyl group forming cap 2. Cap 0 would have only terminal $^{7Me}G(5')ppp(5')X$, (Lewin, 1980).

Synthesis of the 5'Terminal Cap

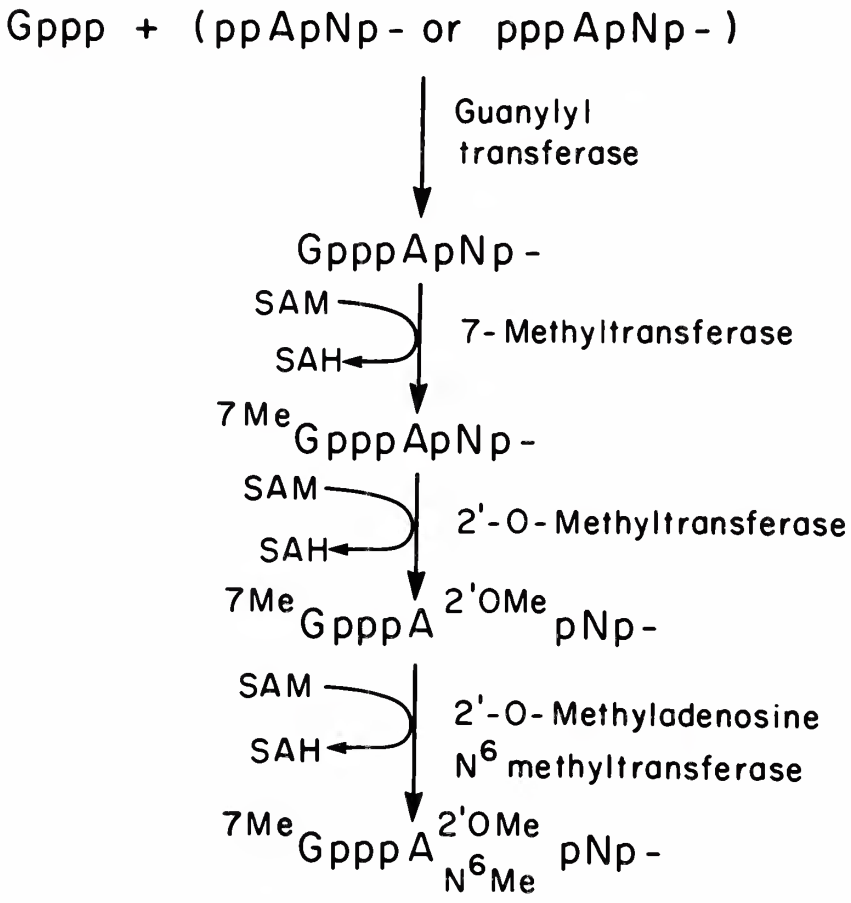
Gppp + (ppApNp- or pppApNp-)
Guanylyl transferase
GpppApNp-
SAM
SAH
7-Methyltransferase
7MeGpppApNp-
SAM
SAH
2'-O-Methyltransferase
7MeGpppA 2'OMe pNp-
SAM
SAH
2'-O-Methyladenosine N6 methyltransferase
7MeGpppA 2'OMe N6Me pNp-

Figure V

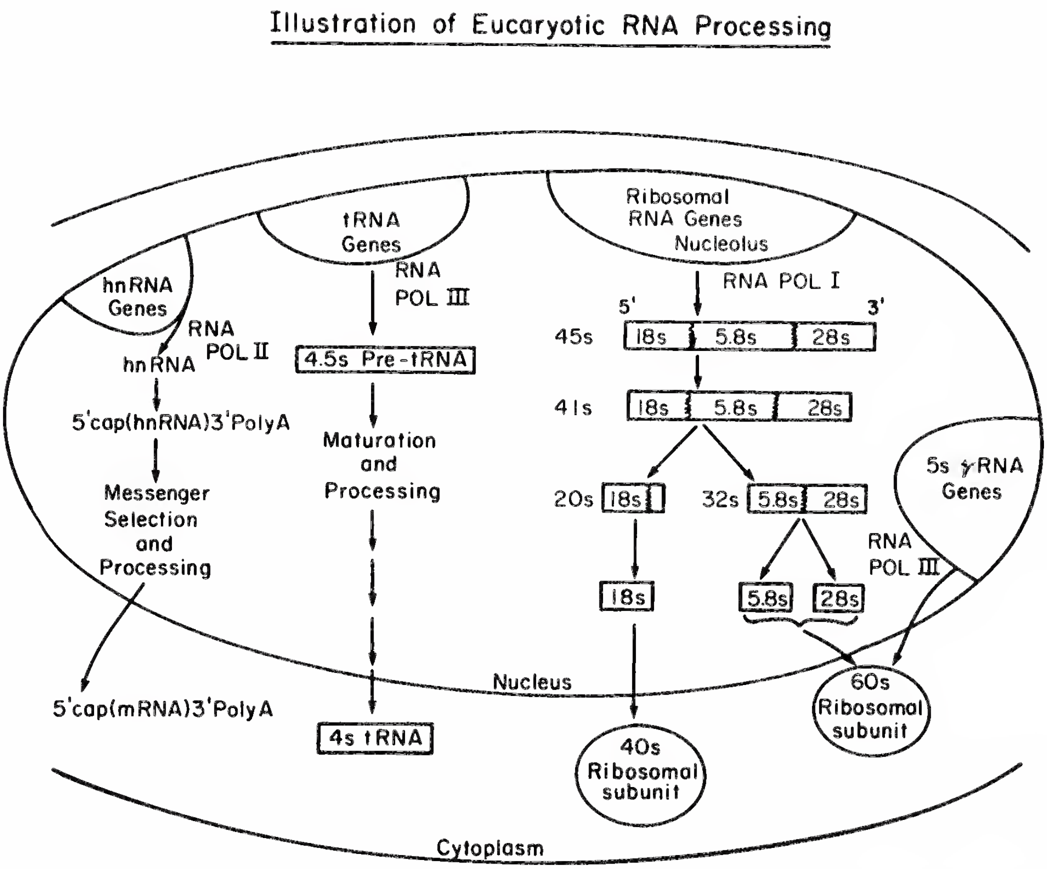
Illustration of Eucaryotic RNA Processing

hnRNA Genes
RNA POL II
hnRNA
5'cap(hnRNA)3'PolyA
Messenger Selection and Processing
5'cap(mRNA)3'PolyA
tRNA Genes
RNA POL III
4.5s Pre-tRNA
Maturation and Processing
4s tRNA
Ribosomal RNA Genes Nucleolus
RNA POL I
5'
3'
45s
18s
5.8s
28s
41s
18s
5.8s
28s
20s
18s
32s
5.8s
28s
18s
5.8s
28s
5s γRNA Genes
RNA POL III
Nucleus
40s Ribosomal subunit
60s Ribosomal subunit
Cytoplasm

Figure VI

Ribosomal RNA processing sequence as determined in HeLa cells (Lewin, 1980).

Structure of Transfer RNA

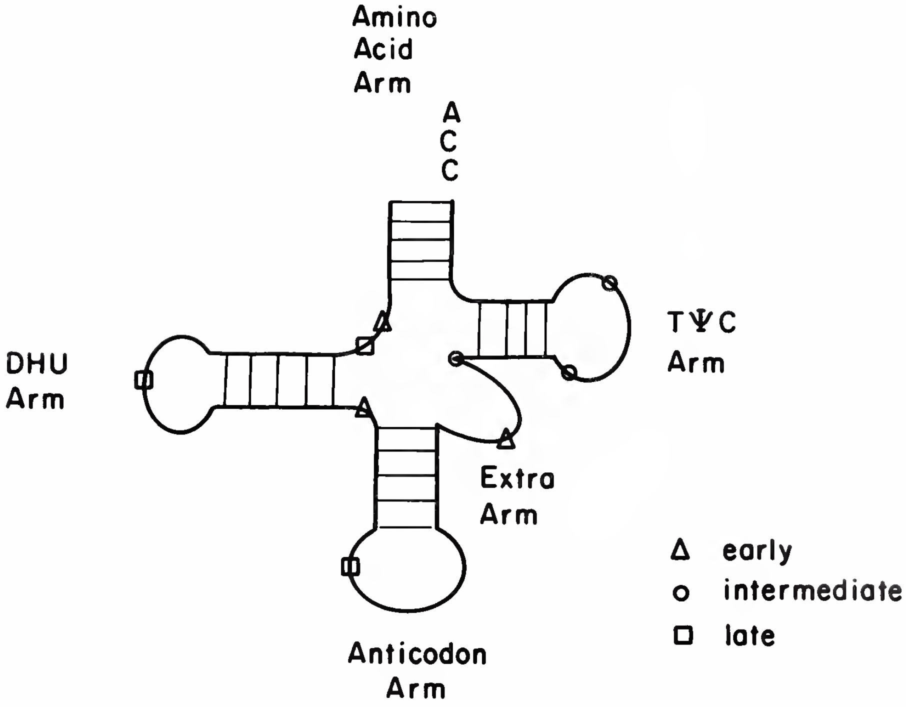
Amino
Acid
Arm
A
C
C
DHU
Arm
TΨC
Arm
Extra
Arm
Anticodon
Arm
Δ early
o intermediate
□ late

Figure VII

Ψ = pseudouridine, DHU = dihydrouridine. Early, intermediate, and late refer to stages of base methylation in tRNA maturation as determined by Munns (1975).

DOSE-RESPONSE OF RNA AND DNA SYNTHESIS BY TUBERCIDIN, 8-AZAADENOSINE AND FORMYCIN

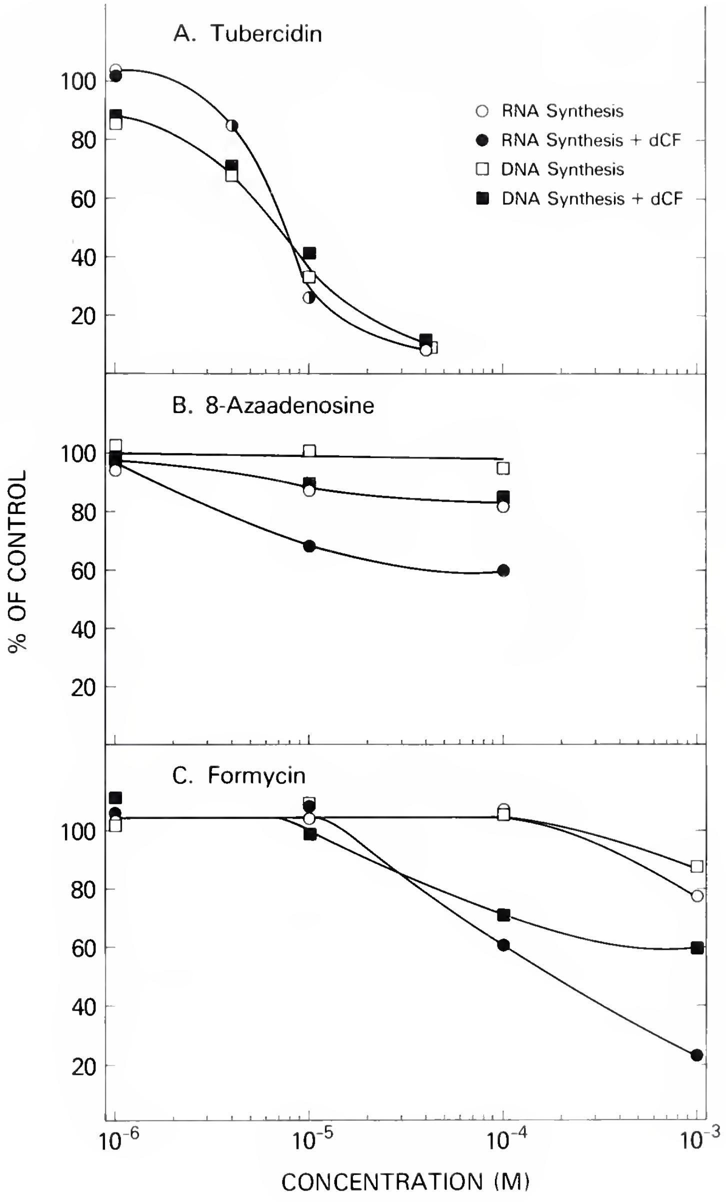

Figure VIII

L1210 cells (1 X 10^7 cells/flask) were preincubated with (●,■) or without (○,□) 1 X 10^{-6}M dCF, and incubated for 30 minutes with tubercidin (A), 8-azaadenosine (B) or formycin (C). Cells were then double-labeled for 1 hour with [^{14}C] uridine (○,●) and [^{3}H] thymidine (□,■) and TCA-precipitable radioactivity was determined as described under Materials and Methods. Each value is the mean of two to three determinations.

DEAE SEPHADEX-UREA CHROMATOGRAPHY OF ALKALINE HYDROLYSATES OF nRNA

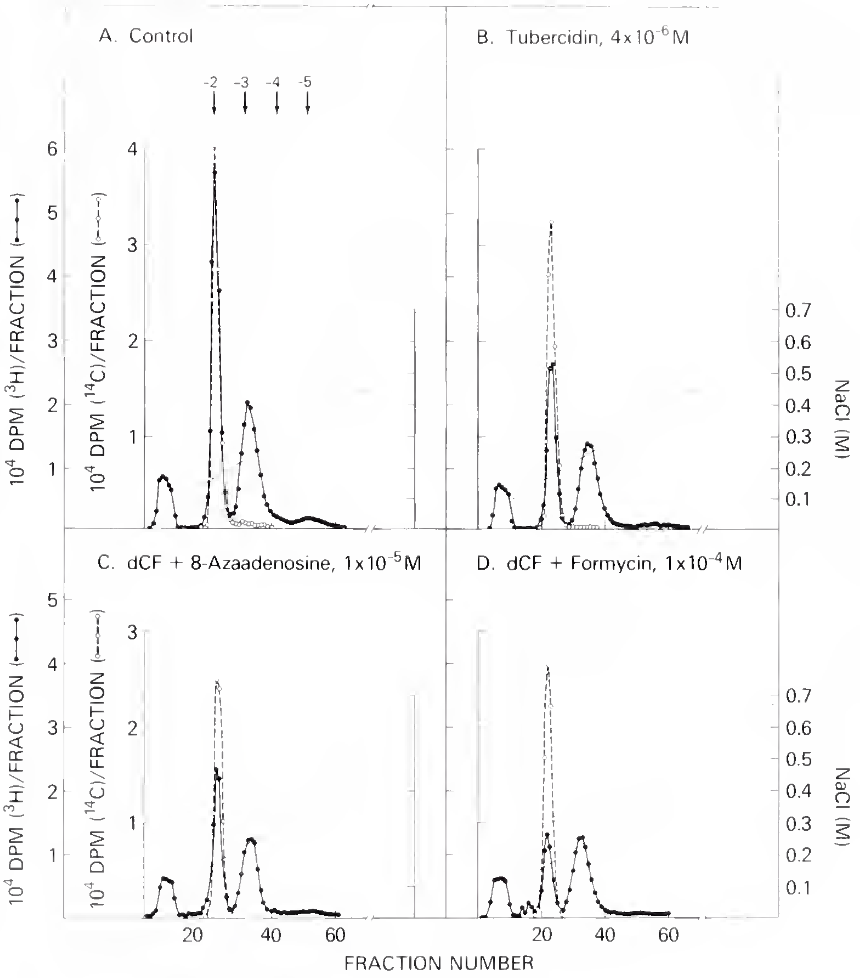

Figure IX

L1210 cells (5×10^7 cells/flask) were preincubated for 15 minutes with (C and D) or without (A and B) 1×10^{-6}M dCF and incubated for 30 minutes with 4×10^{-6}M tubercidin (B), 1×10^{-5}M 8-azaadenosine (C), or 1×10^{-4}M formycin (D). Cells were then double-labeled for 1 hour with 200 uCi L-[methyl-^{3}H] methionine and 5 uCi [^{14}C] uridine. DEAE sephadex-urea chromatography as described under Materials and Methods. The -2, -3, -4, and -5 markers represent the net charge of mono, di, tri, and tetranucleotides, respectively, eluted with the NaCl gradient.

Table 1. DEAE Sephadex-urea chromatography of alkaline hydrolysates of nRNA from L1210 cells treated with tubercidin, 8-azaadenosine and formycin*

	RNA fraction (10^3 d.p.m.)					
	-2		-3		-5	
Treatment	^3H	^{14}C	^3H	^{14}C	^3H	^{14}C
Control	268 ± 28	110 ± 6	186 ± 19	4.15 ± 0.91	20.6 ± 1.7	0.55 ± 0.16
		% of control				
Tubercidin						
$4x10^{-6}M$	59	99	77	53	47	0
$4x10^{-5}M$	9	16	14	2	8	0
dCF+8-Azzadenosine						
$1x10^{-5}M$	45	62	57	42	29	26
$1x10^{-4}M$	50	61	61	43	35	0
dCF+Formycin						
$1x10^{-4}M$	28	73	59	21	26	0
$1x10^{-3}M$	14	38	32	10	14	0

*Values for pooled controls (with and without dCF) represent the means ± S.E. of four determinations. Values for incubations with the lower concentration of each drug represent the mean of two determinations, and at the higher concentration of each drug a single determination. L1210 cells were incubated with tubercidin, dCF + 8-azaadenosine, or dCF + formycin followed by labeling with L-[methyl-^3H] methionine and [^{14}C] uridine as described under Materials and Methods. The fractions -2, -3, and -5 denote the net charge of mono, di, and tetranucleotides of alkaline hydrolysates of nRNA eluted by DEAE Sephadex chromatography depicted in (Fig. IX).

POLYACRYLAMIDE GEL ELECTROPHORESIS OF nRNA

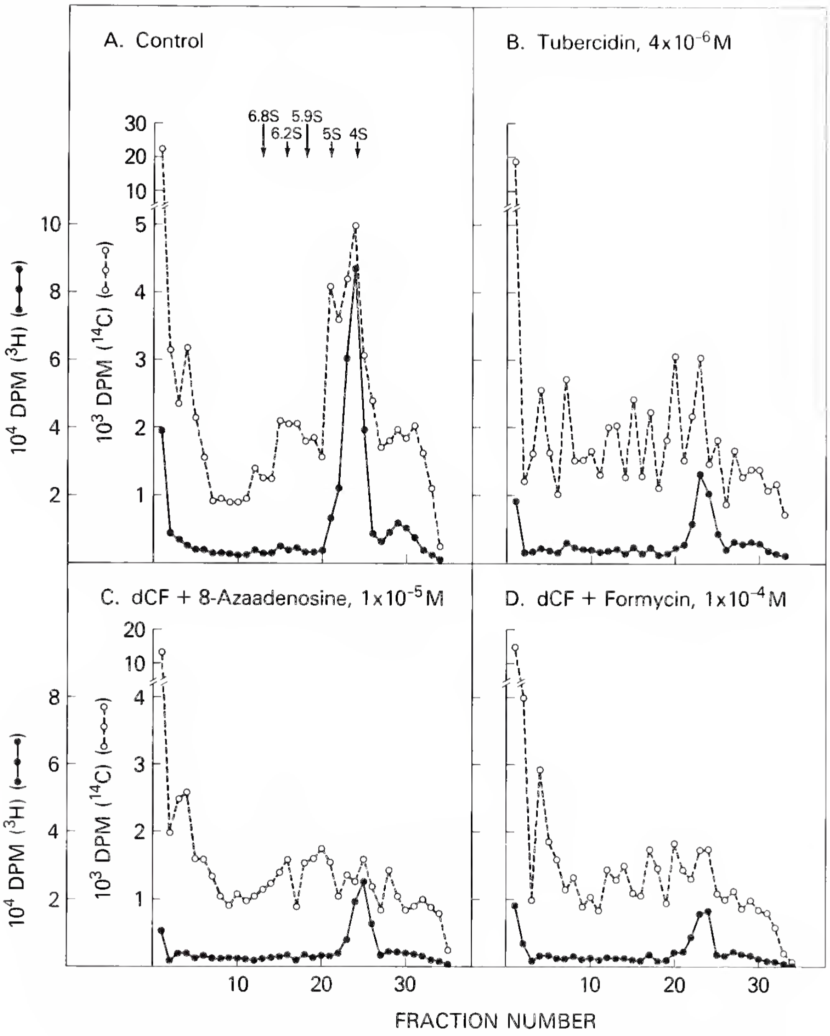

Figure X

L1210 cells (5 X 10^7 cells/flask) were treated as described in (Fig. IX) and electrophoresed in denaturing 8% polyacrylamide-urea gels as described under Materials and Methods.

Table 2. Effects of tubercidin, 8-azaadenosine and formycin on the methylation and synthesis of nRNA from L1210 cells*

Treatment	RNA fraction (% of control)					
	Total		>18S		4SRNA	
	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
Tubercidin						
$4x10^{-6}M$	57 ± 2	85 ± 2	50 ± 5	75 ± 8	43 ± 3	61 ± 8
$4x10^{-5}M$	16 ± 3	8 ± 2	15 ± 3	15 ± 3	14 ± 4	2 ± 1
dCF+8-Azaadenosine						
$1x10^{-5}M$	47 ± 2	62 ± 2	45 ± 4	70 ± 9	38 ± 6	40 ± 10
$1x10^{-4}M$	42 ± 3	63 ± 4	32 ± 3	58 ± 10	30 ± 8	40 ± 12
dCF+Formycin						
$1x10^{-4}M$	33 ± 2	55 ± 2	52 ± 7	57 ± 4	25 ± 1	41 ± 4
$1x10^{-3}M$	27 ± 1	35 ± 1	22 ± 6	32 ± 8	14 ± 3	12 ± 2

*Values represent the means ± S.E. of three to six determinations. L1210 cells were incubated with tubercidin, dCF + 8-azaadenosine, or dCF + formycin followed by labeling with L-[methyl-^{3}H] methionine and [^{14}C] uridine as described under Materials and Methods. Nuclear RNA was isolated and electrophoresed in 8% polyacrylamide-urea gels as depicted in (Fig. X). Radioactivity in > 18S and 4S nRNA was calculated from the radioactivity present at the top of the gel and the 4S region, respectively.

Table 3. Effects of tubercidin, 8-azaadenosine and formycin on the composition of methylated nucleosides in 4S nRNA from L1210 cells*

	Methylated nucleoside (d.p.m.)							
Treatment	m^7G	m^5C	m^3C	m^2G	m^2_2G	m^1G	m^5U	m^1A
Control	3230	2220	630	4590	4290	2520	3430	300
			% of control					
Tubercidin $4 \times 10^{-6}M$	20	54	27	32	36	28	41	88
dCF+8-Azaadenosine $1 \times 10^{-5}M$	47	42	27	55	50	42	53	93
dCF+Formycin $1 \times 10^{-4}M$	25	9	40	30	17	19	31	83

*Each value is the mean of two determinations. Thin-layer chromatography and determination of radioactivity as described under Materials and Methods.

Eucaryotic Metabolism of S-Adenosylmethionine and S-Adenosylhomocysteine

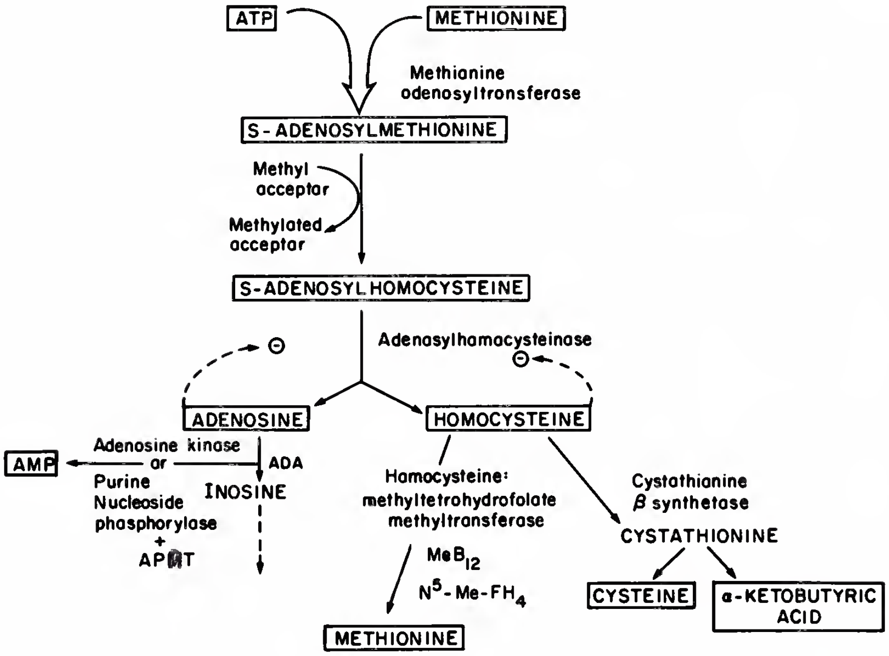
ATP
METHIONINE
Methionine adenosyltransferase
S-ADENOSYLMETHIONINE
Methyl acceptor
Methylated acceptor
S-ADENOSYLHOMOCYSTEINE
Adenosylhomocysteinase
⊖
⊖
ADENOSINE
HOMOCYSTEINE
AMP
Adenosine kinase
or
Purine Nucleoside phosphorylase
+
APRT
ADA
INOSINE
Homocysteine: methyltetrahydrofolate methyltransferase
MeB_{12}
N^5-Me-FH_4
METHIONINE
Cystathionine β synthetase
CYSTATHIONINE
CYSTEINE
α-KETOBUTYRIC ACID

Figure XI

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