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Effects Of 5-Fluorouracil On Escherichia coli Ribosomes

A Thesis

Presented to the

Department of Biology

and the

Faculty of the Graduate College

University of Nebraska

In Partial Fulfillment

of the Requirements for the Degree

M.A. in Biology

University of Nebraska at Omaha

by

Susan P. Belleh

November 1995

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ACCEPTANCE PAGE

Acceptance for the faculty of the Graduate College, University of Nebraska, in partial fulfillment of the requirements for the degree (M.A. in Biology), University of Nebraska at Omaha.

Committee

Name	Department/School
[signature]	Eppley Institute UNMC
Lena A. Chase	Biology UNO

Chairperson William E Jaggrich

Date Nov 16, 1995

ABSTRACT

5-Fluorouracil (FUra), a chemotherapeutic agent, affects the growth of several kinds of tumor cells. It is known to affect DNA as well as RNA. Using *Escherichia coli,* this study has shown that FUra interferes with the synthesis and assembly of ribosomes. Cells treated in FUra concentrations between 25 ug/ml and 50 ug/ml gave severely retarded growth rates. Sucrose density gradient absorbance profiles of ribosomes from FUra-treated cells indicated altered structural and functional properties. These profiles showed large pools of free ribosomal subunits and few completely associated ribosomes or polyribosomes. The 23S rRNA from FUra-treated ribosomes showed an increased amount pf degradation, consistent with previous studies. However, the 16S rRNA of the 30S ribosomal fraction showed no evidence of breakdown products. Composite gel electrophoresis of FUra-treated samples indicated a decrease in S1 protein, a factor which is vital for translation. Analysis of ribosomal proteins revealed that most proteins were present in normal stoichiometries. However, some non-ribosomal proteins associated with unfolded ribosomal particles were present in samples with higher concentrations of FUra. Incorporation studies, showed that ^{3}H-FUra was efficiently incorporated into cells and ribosomes. Incorporation into 30S and 50S subunits was equivalent, but with increased exposure, increasing amounts were incorporated into the translating pool of ribosomes (70S and polyribosomes). The absorbance profiles also showed more assembled ribosomes and polyribosomes with increasing exposure, suggesting that a transient effect on some cellular factor, may have been overcome.

ACKNOWLEDGEMENTS

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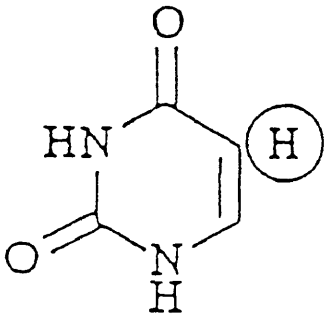
INTRODUCTION

5-fluorouracil (FUra) is a drug that has been used extensively as a chemotherapeutic agent in the treatment of adult cancers and solid tumors including colorectal (Mandel, 1979), breast (Ansfield et al., 1969) and liver carcinomas. While the drug is effective in treating cancer, it is also toxic when given in high doses. The mechanism of action of FUra has been studied for over 30 years to understand why it is an effective chemotherapeutic drug. Despite these studies, information concerning the specific cellular targets of the compound remains fragmentary.

Early investigations into nucleotide metabolism indicated that cancer cells utilized more uracil than did normal cells. Specifically, chemically induced rat hepatoma cells incorporated greater quantities of uracil than did normal rat liver cells (Rutman et. al., 1954). Since rapidly dividing cells showed an enhanced incorporation of uracil into nucleic acids, it was suggested that an antimetabolite similar to uracil in structure, but different in function could be utilized as a specific tumor cell inhibitor (Heidelberger, 1975). Among the potential uracil analogs, the substitution of fluorine, an atom more electronegative than hydrogen (but similar in size), at position 5 within the uracil molecule was found to be very stable, amenable to chemical synthesis (Wempen and Fox, 1964) and did not interfere with the utilization of the base, nucleoside, or nucleotide in cellular metabolism. Thus, FUra was manufactured for study as a chemotherapeutic agent.

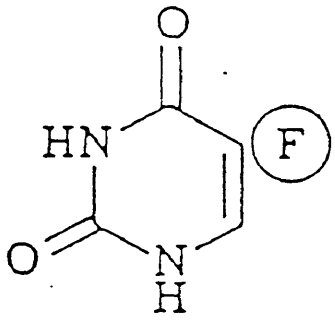
FUra is a fluorinated analog of uracil (Figure 1). Several cellular targets



Uracil

H = 1.20 Å

pK_a = 9.45



5-Fluorouracil

F = 1.35 Å

pK_a = 8.15

Figure 1. Structural and physical similarities between 5-fluorouracil and the natural pyrimidine uracil. Substitution of hydrogen at position 5 of the uracil molecule for fluorine, a more electronegative atom, produced a stable structurally similar to uracil, stable, and amenable to chemical sythesis (Heidelberger, 1975).

have been suggested for the drug. These include the metabolic pathways of thymidine synthesis, cellular targets such as messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), small nuclear RNAs (snRNA) and deoxyuridine and thymidine nucleotides in DNA.

FUra METABOLISM

The structural similarity of FUra to the natural pyrimidine, uracil, allows the drug to enter the nucleotide biosynthesis pathways (Mandel et. al., 1979). The metabolic pathway of FUra has been studied extensively (Parker and Cheng, 1990) (Fig. 2). It can be metabolized to 5-Fluorouridine (FUrd), FUrd 5'-triphosphate (FUTP), 5-Fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP), and 5-Fluoro-2'-deoxyuridine-5'-triphosphate (FdUTP) compounds which have different mechanisms of action depending on their metabolism in different cell lines. One very important part of the metabolic pathway of FUra is the conversion of 5-Fluoro-2'-deoxyuridine (FdUrd), a compound similar to FUra, to FdUMP by the action of thymidine kinase. This results in DNA-directed cytotoxicity of fluoropyrimidines. Little or no incorporation into RNA occurs via this pathway. Effects on RNA can only be observed when FUra is converted to FUrd or when FdUrd is converted back to FUra by cellular phosphorylases. In whole animals, FUrd and FdUrd are converted to FUra by uridine and thymidine phosphrylases respectively. Hence, treatment of whole animals with either FdUrd or FUrd is the same as treatment with FUra and both RNA- and DNA-directed cytotoxicity are observed.

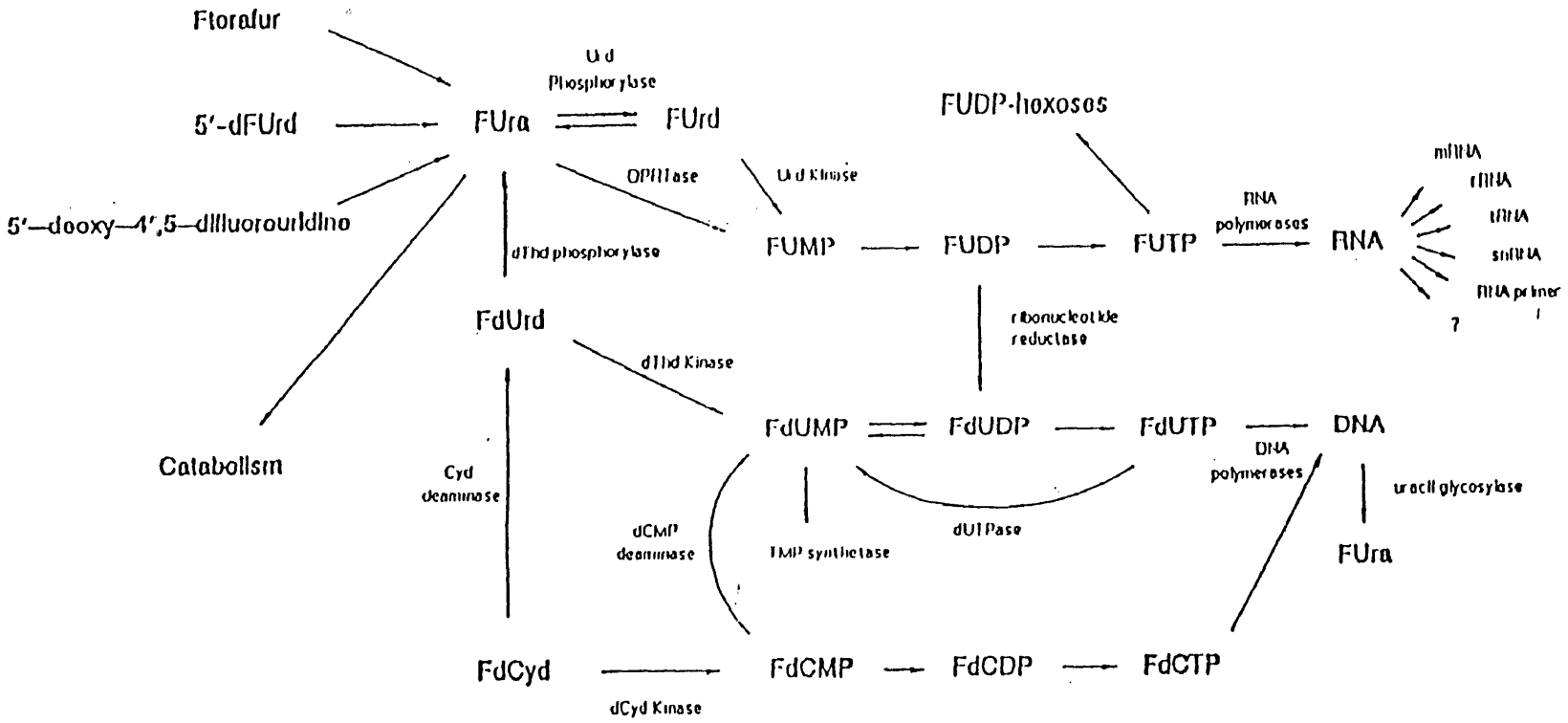


Figure 2. Metabolic pathways of fluoropyrimidines leading to incorporation into DNA and several RNA species (Parker and Cheng, 1990).

EFFECTS OF FUra ON THYMIDYLATE SYNTHASE AND DNA SYNTHESIS

In DNA, thymine (5-methyluracil) replaces the uracil found in RNA. The synthesis of thymidine monophosphate (dTMP or thymidylate) is essential to provide the thymidine triphosphate (dTTP) needed for DNA replication. Thymidylate is synthesized from dUMP by two pathways. One pathway involves the conversion of dCMP by deoxycytidylate deaminase, an enzyme that is widely distributed in animal tissue, to dUMP (Zubay, 3rd edition).

$$dCMP + H_2O \rightarrow dUMP + NH_3$$

The other pathway involves the phosphorylation of dUDP to dUTP which then becomes hydrolyzed to dUMP. This route to dUMP is more widely utilized because ribonucleotide reductase, an enzyme that catalyzes the reduction of the ribose ring of ribonucleotides in eukaryotic and some prokaryotic cells, only acts on ribonucleoside diphosphates. This aids in the regulation of nucleotide metabolism in most cells. In addition, cells contain deoxyuridine triphosphate diphosphohydrolase (dUTPase) which prevents the incorporation of dUTP into DNA by keeping dUTP levels within the cell low (Figure 2). Once dUMP is

$$dUTP + H_2O \rightarrow dUMP + PPi$$

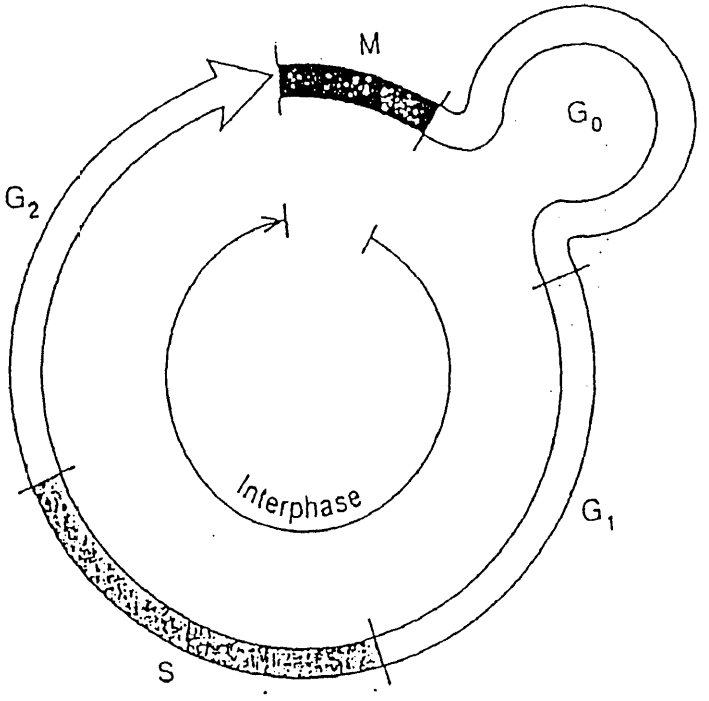
produced, it is methylated by utilizing 5,10-methylenetetrahydrofolate to give thymidylate (dTMP). The enzyme thymidylate synthase (TS) catalyzes the

reaction. This is a unique reaction because the folate derivatives acts as both the donor and the reductant using the pteridine ring as its source of the reducing potential (Zubay, 3rd edition).

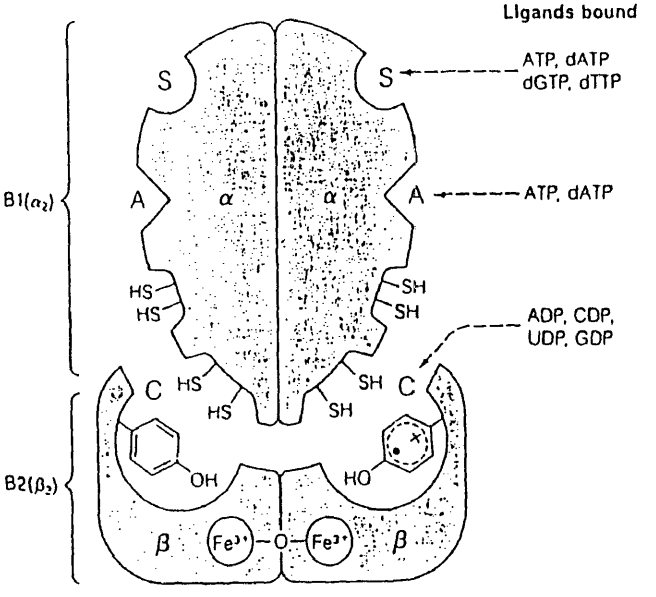
Studies have shown that FUra and other fluorinated pyrimidines enter these pathways and become incorporated into DNA. Once incorporated, the fluorinated compounds cause DNA fragmentation, (Caradonna et al., 1980), lead to mutation (Aebersold, 1979), and also inhibit DNA replication (Fernandes et al., 1986).

Since DNA synthesis is not continuous throughout the cell cycle, effects on DNA are dependent in part, upon the phase of cell cycle. In *E. coli* cells, DNA replication occurs only once during its 30 min cell cycle. In eukaryotic cells, DNA synthesis occurs during one phase of the cell cycle, the S phase (Fig. 3a). A gap of time (G2) occurs after DNA synthesis but before cell division (M). Another gap of time (G1) occurs before DNA synthesis after mitosis. Many cells rest after mitosis and exit the cycle into the G0 state and become quiescent. In most eukaryotic cells, growth is a carefully regulated process. The duration of the entire cell cycle varies from cell type to cell type. A single decision made in the cell cycle dictates when a cell leaves G1 and becomes committed to DNA replication in the S phase. Once this decision has been made, many events occur, causing the cell to enlarge, duplicate DNA, segregate each DNA molecule to separate nuclei and finally divide into two daughter cells (mitosis).

Normal cells going through the cell cycle have kinases and other regulatory molecules that control the timing of events of the cell cycle. These



(a)



(b)

Figure 3.
(a) Cell cycle of a eukaryotic cell showing the 5 phases (Zubay, 1990). G0 - the resting stage of the cell before it is triggered to enter G1 - prereplication stage. Certain conditions occur for cells to enter the S stage - replication of DNA. Cells leave S to enter G2 - the postreplication stage before entering the mitotic stage of the cell cycle.
(b) Model of *E.coli* ribonucleotide reductase (Mathews & van Holde, 1990).

factors regulate the level and activity of nucleotide metabolism enzymes like thymidylate synthase and ribonucleotide reductase. The levels and activity of these enzymes must increase when the cell prepares to enter the S phase of the cell cycle. This occurs under three different circumstances; when the cell prepares to leave the resting phase (G0) of the cell cycle, when it performs extensive repair after DNA damage, and after an infection by a virus or any other mitogen that would cause quiescent cells to proceed into the S phase. These two enzymes subsequently decrease in activity once DNA synthesis is completed (Zubay, 3rd edition).

To arrest normal cell growth, removal of one or more deoxyribonucleotides is necessary. The removal of thymidine or very low concentrations of thymidine causes cells to go into arrest. Complex allosteric effects on ribonucleotide reductase can help to explain the role of nucleotide levels on cell cycle. Accumulating dTTP binds to the specificity sites of ribonucleotide reductase causing dCTP levels to decrease while the concentration levels of dATP, dGTP and dTTP increase. Since ribonucleotide reductase is controlled in both activity and specificity, this enzyme can maintain a balanced pool of DNA precursors (Figure 3b). If ATP is bound to the active site, catalytic activity on ribonucleotides increases. The opposite occurs when dATP is bound. Nucleotides bound to the specificity sites of the enzyme cause it to maintain a balanced rate of the production of all four dNTPs. Tumor cells lack this effective control on cell growth by nucleotide biosynthesis enzymes. Consequently, thymidylate synthase and ribonucleotide reductase are continually active, allowing cell growth and DNA synthesis to continue

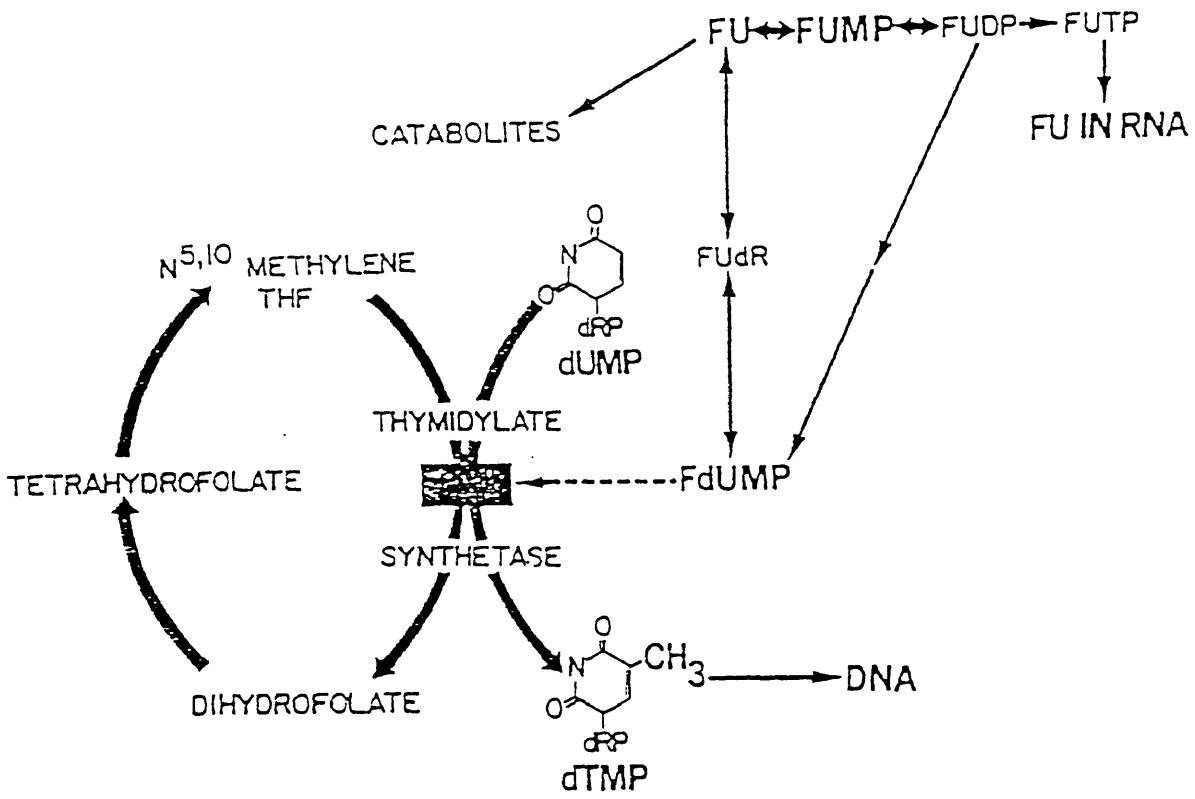


Figure 4. The effects of FUra on DNA synthesis. FUra is metabolized to FdUMP which blocks the action of thymidylate synthase in the presence of 5, 10-methylentetrahydrofolate. It inhibits the action of thymidylate synthase (TS) to methylate dUMP to dTMP, necessary for DNA synthesis (H. Mandel *et. al.*, 1978).

uncontrollably.

One mechanism by which FUra inhibits tumors involves the inhibition of thymidylate synthase (TS). By inhibiting TS, 5-fluorouracil blocks DNA synthesis (Figure 4). In the presence of the methyl donor 5, 10-methylenetetrahydrofolate, FUra inhibits the reaction catalyzed by thymidylate synthase, which converts dUMP to dTMP (Takimoto et al., 1993). Since folate derivatives are maintained at very low levels in cells, continuous regeneration of 5, 10- methylenetetrahydrofolate is necessary for continuous synthesis of thymidylate. Thus, DNA synthesis can be inhibited either by blocking thymidylate synthase or dihydrofolate reductase. The metabolite that is most likely responsible for the effects of FUra on DNA synthesis is 5-fluorodeoxyuridine 5'-monophosphate (FdUMP). Once synthesized, FdUMP binds covalently to thymidylate synthase and blocks thymidine formation. In this way, DNA synthesis is inhibited in normal cells and in tumor cells. This leads to undesirable cell death in normal cells growth control and / or death in cancer cells.

Given the activity of FdUMP on TS, it was widely accepted that FUra mediated cytotoxicity in tumor cells was through the inhibition of DNA synthesis. Recently however, studies have shown that this is not the sole mechanism (Greenhalgh and Parish, 1989; Akazawa et. al., 1986). Administration of thymidine (TdR) to FUra-treated cells to revert the cytotoxic effects of FUra on DNA synthesis enhanced rather than decreased the cytotoxicity of the drug. At the same time, incorporation of FUra into RNA was markedly increased. This was explained by the assumption that FUra metabolized to FdUTP as a result of

the action of ribonucleotide reductase. Then the addition of TdR to revert the action of FdUMP on thymidylate synthase suppressed FdUTP production (Figure 4) (Akazawa et. al., 1986). It also led to an increased amount of dTTP in these cells causing a feedback inhibition of ribonucleotide reductase and consequently inhibition of normal cell growth (Speigelman et. al., 1980). The high dTTP levels in these cells may have repressed the anabolic conversion of FUra into deoxyderivatives, thus allowing the drug to become incorporated into RNA more readily (Ghosal and Jacob, 1994, Speigelman et. al., 1980). Interestingly, Greenhalgh and Parish (1989) found that addition of TdR not only enhanced the incorporation of FUra into total cellular RNA, but it also disrupted rRNA processing.

As a result of this and similar studies showing that FUra cytotoxicity is RNA-mediated, several investigators have turned their focus to analyzing the effects of FUra on cellular RNA molecules. Ultimately, understanding the mechanism of FUra treatment on both RNA and DNA, and its effectiveness as a chemotherapuetic agent will enhance the clinical management of various solid tumors.

RNA SPECIES

Three kinds of cellular RNA are involved in protein synthesis. Messenger RNA (mRNA) carries the information transcribed from DNA for polypeptide synthesis. Transfer RNA (tRNA) carries amino acids to the mRNA template for protein synthesis. Ribosomal RNA (rRNA) is a structural and functional component of ribosomes. Of the three kinds of RNA involved in

protein synthesis, this present study has considered the effects of FUra on rRNA.

EFFECTS OF FUra ON RNA SPECIES

FUra and FUrd are metabolized into different uracil triphosphate (UTP) pools leading to their incorporation into several different RNA species (Takimoto et.al., 1986; Shani and Dannenberg, 1984). Over the past 10 - 15 years, several hypotheses have attempted to explain the RNA-mediated cytotoxicity of FUra (Glazer et al., 1979, 1980; Tseng et al., 1978; Wilkinson et al., 1975). While a correlation between the ability of FUra to become incorporated into RNA and the FUra-directed cytotoxicity has been observed in many cases (Cadman et al., 1979; Spiegelman et al., 1980), the mechanism by which this occurs is still a mystery.

FUra EFFECTS ON MESSENGER RNA (mRNA)

FUra substitutes for uracil in all types of RNA to cause changes in RNA metabolism (Wilkinson et al., 1973; Takimoto et al., 1987). Takimoto et. al., (1993) showed that FUra-substituted mRNA produced identical levels of active protein as those produced by the control mRNA. Their results showed that the *in vitro* translational products of FUra-substituted and control mRNAs were identical. There was no evidence of miscoding due to FUra incorporation into mRNA. Similar results were produced earlier by Grunberg-Manago et. al. (1964). Since FUra has been shown to have no effect on the translational products of mRNA, any effects on mRNA must occur at the the level of nuclear

mRNA processing (Takimoto et. al., 1993).

FUra EFFECTS ON SMALL NUCLEAR RNA (snRNA) AND SPLICING

Incorporation of FUra has been shown to produce alterations in certain small nuclear RNAs (snRNAs) and may contribute to alterations in mRNA processing, or localization of mRNA in nuclear compartments (Cohen et al., 1985; Berget, 1984). Small nuclear RNAs (snRNA) are molecules that participate in splicing reactions of mRNA. Splicing involves the removal of introns within the mRNA molecule and sealing together the ends of the exons. RNA splicing has been studied intensely in yeast (Figure 5) (Konarska and Sharp, 1987).

There are six prominent snRNAs, U1 - U6. They all associate in the nucleus along with 6-10 ribonucleoproteins to form spliceosomes (snRNPs) (Steitz et. al., 1988). Armstrong et. al. (1986), demonstrated that the incorporation of a 10 uM concentration of another fluoropyrimidine, FUrd, affected three different snRNAs. FUrd was the most potent fluoropyrimidine capable of producing RNA-mediated cytotoxicity related to snRNA metabolism in murine tumor cells. The results suggested that FUra incorporation altered the secondary structural properties of U4 and U6 snRNAs and reduced the turnover rate of the U1 snRNA. For U4 and U6, a high percentage of FUrd substitution in a hairpin loop of these snRNAs caused the disruption of the secondary structure and subsequently inhibited cellular mRNA processing. However, these changes were only observed at very high FUrd concentration (10 uM) suggesting that the*in vitro* effects on snRNA cannot account for *in vivo*

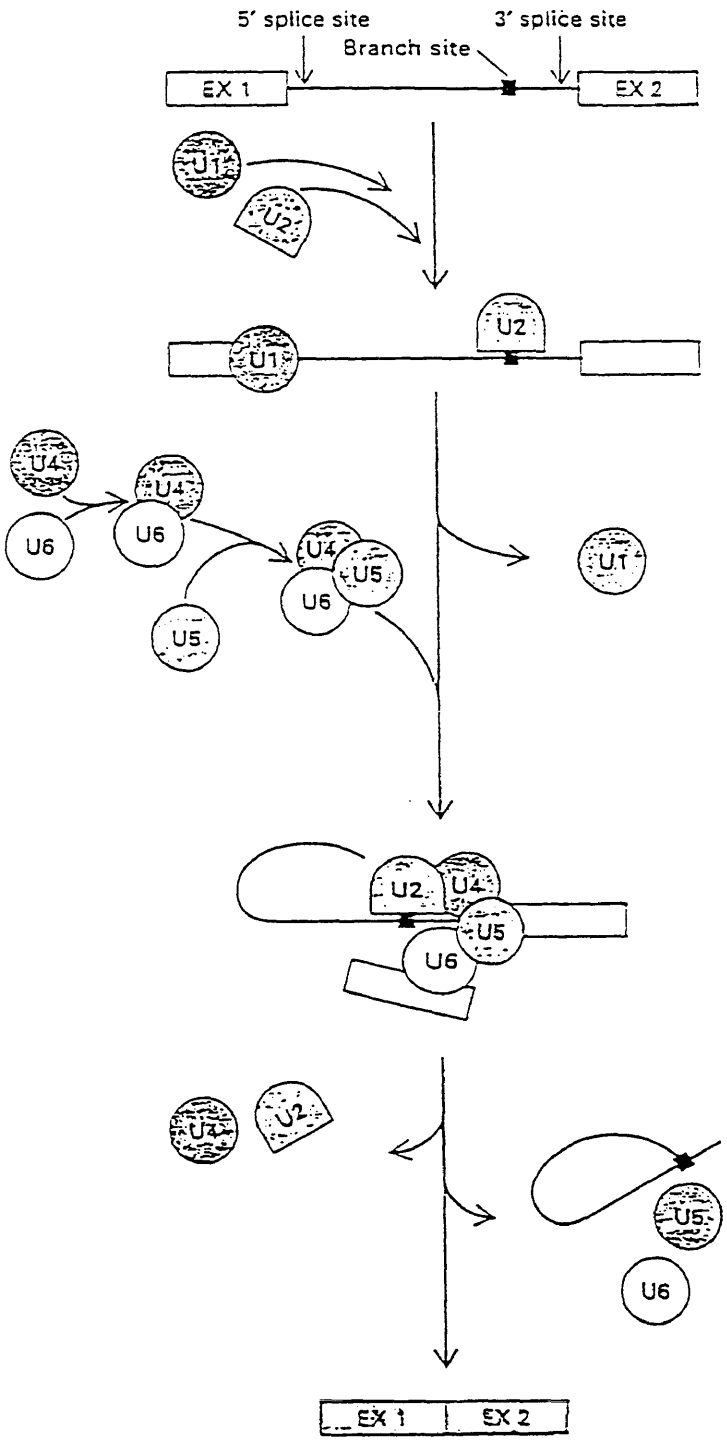


Figure 5. Pathway of spliceosome assembly as studied in yeast (Darnell *et. al.*, 1990).

cytotoxicity. To minimize the in vitro effects, high concentrations of FUrd would have to be maintained for a long period of time.

In the case of the reduced turnover of U1 in these cells, normal processing of nuclear mRNA could indeed be hampered. Without U1, the 5' splice site of the mRNA is not cleaved and the 5' splice site of exon 1 cannot be brought into position to become ligated to the 3' splice site of exon 2 (Figure 5). Thus, reduced turnover of the U1 snRNA leads to reduced mRNA processing. All of these changes correlate well with the cell viability of FUra-treated cells suggesting that changes in snRNA metabolism may suppress the normal relocation of mRNA from the nucleus to the cytoplasm (Berget, 1984). It may also contribute to the altered molecular weight of RNA in fluoropyrimidine-treated cells (Armstrong et. al., 1986).

Similar studies in cells depleted of the U2 and U6 snRNAs showed that splicing activities were decreased in cells using FU2 and FU6 (Heinz-Josef Lenz et. al., 1994). In this particular study, the U2 snRNA was more sensitive than U6 to FUra treatment. FUra substitution in U2 snRNA altered protein recognition sites and as a result, lowered the stability of the base pairing in a stem loop structure of this snRNA. The weakened binding of splicing factors promoted the formation of an uncharacterized complex that increased the degradation observed in the pre-mRNA molecule. Normally, splicing factors such as SnRNPs are bound to U2 precursor mRNA molecule thereby protecting them from hydrolysis. Thus, the complex formed by FU2 was not on the normal pathway of spliceosome assembly. In the case of U6, high concentrations of FU6 were necessary to restore full splicing activities to the U6-depleted cell

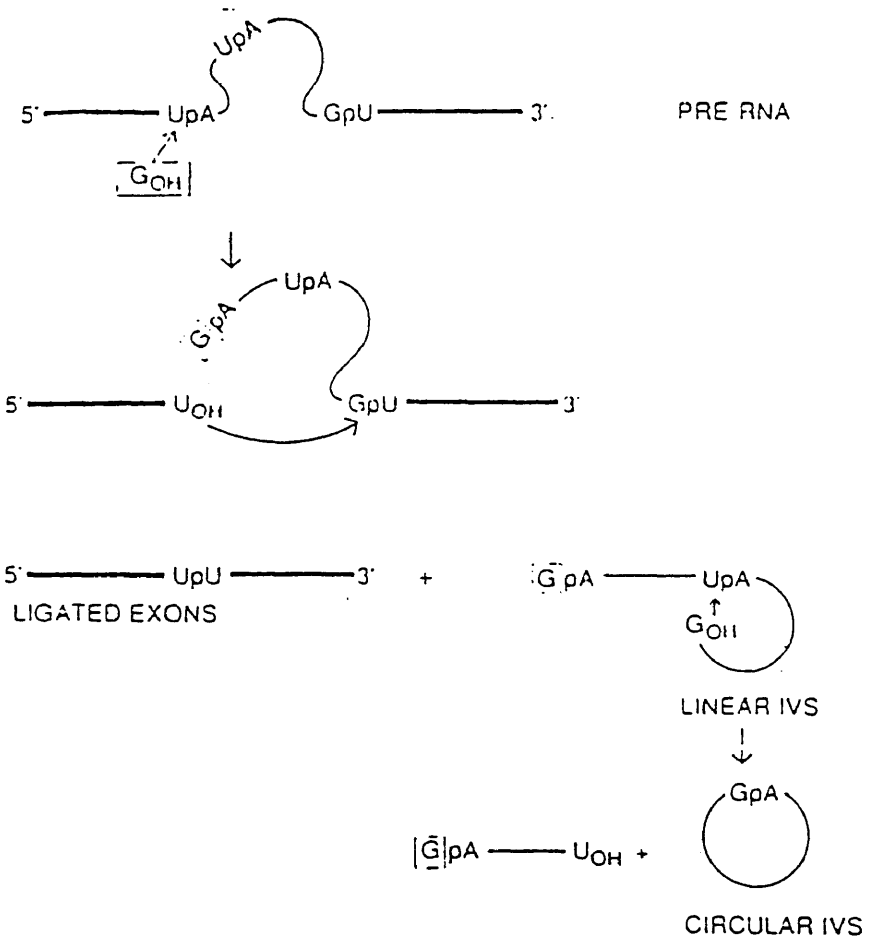


Figure 6. Mechanism of self-splicing pre-rRNA sequence in *Tetrahymena thermophilia*. The rRNA has two exons and a 408 bp intron. Splicing of the rRNA occurs by the removal of the intron by the cleavage at the 5' exon-intron junction in the presence of a guanosine (GOH) nucleotide. Once it is cut, the free 3' hydroxyl of the guanosine molecule becomes linked to the 5' phosphate of the nucleotide at the 5' phosphate of the nucleotide at the 5' end of the intron Transesterification (tranfer of a phosphate linkage from one nucleotide to another) occurs at both ends of the intron. The second cleavage (at the 3' end) occurs during transesterification that links the end nucleotides in a 5'-3' phosphodiester linkage. Linkage is provided by the final UMP of the 3' exon (Danenberg *et. al.*, 1990).

extracts. Overall, the study suggested that FUra incorporation into U6 resulted in a lower stability in the FUra=A base pairing which may facilitate a dissociation of FU6 from U4. This would inhibit catalytic steps involved in spliceosome assembly (Figure 5). However, a recent study done by Gmeiner et. al., (1994) shows that the FUra=A base pairs are more stable than U=A base pairs.

Another splicing mechanism in *Tetrahymena thermophila*, was also examined in the presence of FUra. In this case, the transcribed pre-rRNA molecule undergoes a splicing reaction in the absence of cellular proteins, but in the presence of guanosine which donates the chemical activity of a 2' hydroxyl group (Darnell et. al., 1990) (Figure 6). The formation of splicing products in this reaction was monitored in the rRNA of FUra treated cells. Danenberg et. al. (1990) showed that FUra inhibits the self splicing process in *Tetrahymena* rRNA even though no abnormal splicing products were observed. The inhibition was explained again by the lower stability of base pairing between FUra=A pairs compared with normal U=A pairs (Takimoto et al., 1993). Taken together, there is ample evidence to suspect that FUra can block processes involved in mRNA processing.

FUra EFFECTS ON TRANSFER RNA (tRNA)

The tRNA molecule decodes the information on the mRNA so that the appropriate amino acid is added to the polypeptide chain. Each kind of tRNA molecule is recognized by a particular amino acyl tRNA transferase. There are more than 20 tRNA's but only 20 tRNA amino acyl transferase. Within the

primary structure of the tRNA molecule are modified bases such as dihydrouridine (D), inosine (I), thymine (T), pseudouridine (Ψ) and methylated bases (M) (Figure 7).

Transfer RNA structure has also been investigated in the presence of FUra to address whether FUra substituted tRNA contributes to altered cell growth. Data of Tseng et. al. (1978) indicate that FUra decreases the activity of tRNA uracil-5-methyltransferase in the liver and in tumor cells of FUra-treated mice *in vitro*. Tumor cells generally show increased activity of tRNA methyltransferase compared to normal cells (Wilkinson and Crumley, 1977). Tseng's data indicate that the activity of tRNA uracil-5-methyltransferase (U^5MeU) was reduced. This effect is similar to the inhibition of thymidylate synthase (TS) by FdUMP (Figure 4). As previously mentioned, FdUMP binds covalently to TS. Tseng et. al, believes that there may be an unidentified drug metabolite that binds tightly or covalently to the tRNA uracil-5-methyltransferase. *In vitro* assays of tRNA U^5MeU in the presence of 2 mM FUra showed that FUra was a much weaker inhibitor than uracil itself, suggesting that a metabolite other than FUra is the inhibitor of U^5MeU. The study also showed that FUra exhibited a preferential inhibitory effect on tumor cell tRNA. This may partially contribute to the antineoplastic actions of FUra.

FUra is also known to specifically inhibit m^5U formation in tRNA, without interfering with other methylated bases within the molecule. Tseng et. al., (1978) also found that there was a reduction in the synthesis of pseudouridine (U) and dihydrouridine (D) bases due to FUra incorporation. When these

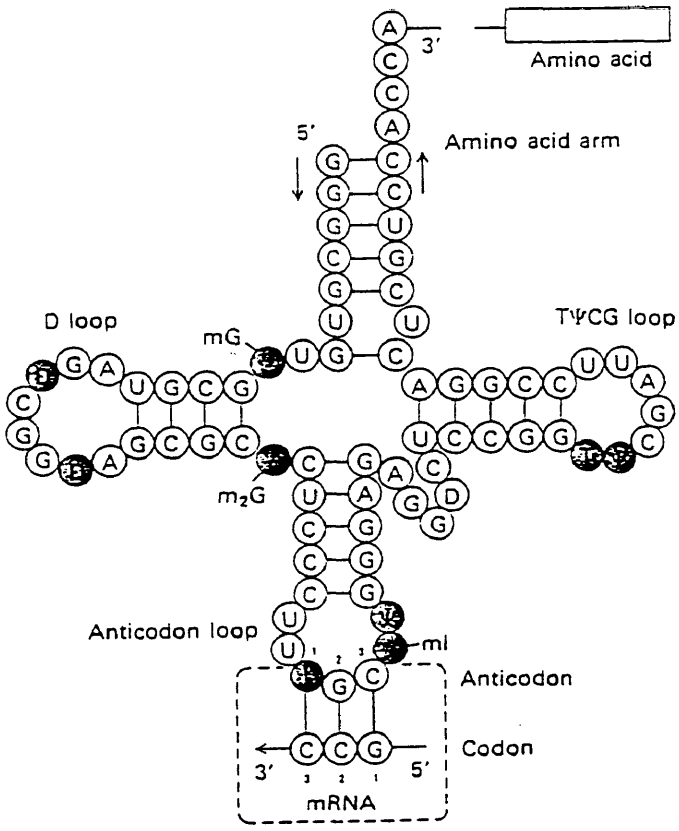


Figure 7. The primary structure of yeast alanin tRNA (tRNA ^{Ala}) is a cloverleaf. It is synthesized from the usual nucleotide bases A,C,G,U. The modified bases are D = dihydrouridine, I = Inosone, T= thymine, Ψ = pseudouridine, and m = methyl group. It has four base pair stems; the anticodon loop, the D loop, the TΨCG loop (for thymidylate, pseudouridylate, cytidylate and guanylate which are present almost all the time in this loop) (Darnell et. al., 1990).

reviewed by Heidelberger et. al., (1983), it was suggested that incorporation into tRNA does not play a major role in RNA-mediated cytotoxicity of FUra since protein synthesis is not affected (Dolnick and Pink, 1985).

RIBOSOMAL RNA AND PROTEINS

Ribosomal RNA is part of the machinery that is associated with protein synthesis. The 70S ribosome of prokaryotes is made up of two subunits, the 50S and the 30S subunits (Figure 8). They are both composed of RNA and protein. The 50S subunit contains the 23S and 5S rRNAs, which contains 2904 and 120 nucleotides respectively. They are complexed with 33 proteins (L1 - L33). The 30S subunit is composed of the 16S rRNA containing 1542 nucleotides and 21 proteins (S1 - S21) (Zubay, 3rd Ed.). Eukaryotic ribosomes are constructed in a similar manner, but the RNA molecules are larger and they have more proteins.

The primary function of ribosomal proteins is to bind and assemble individual domains of rRNA into their appropriate positions (Zubay, 3rd Ed.). Studies have determined a pathway by which individual proteins bind to rRNA in an orderly sequence. The assembly maps for both 30S and 50S subunits show that the assembling ribosome undergoes conformational changes at specific points along the pathway (Figure 9). Once assembled into mature subunits, rRNA participates in all stages of protein synthesis; initiation, elongation and termination (Figure 10).

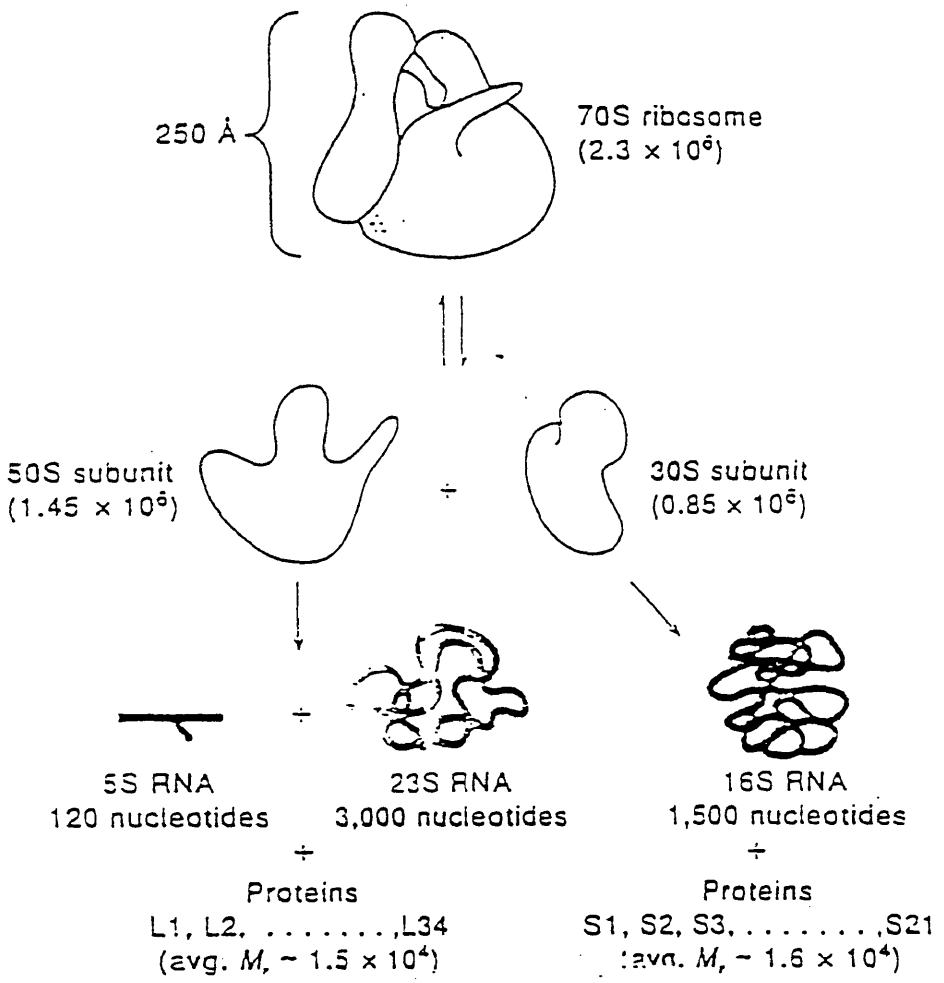


Figure 8. Composition of *E. coli* ribosomes. The 70S complex is made up of the 50S and 30S subunits which become associated during translation. Each subunit contains ribosomal protein and RNA molecules (Zubay, 3rd Ed.).

FUra EFFECTS ON RIBOSOMAL RNA (rRNA)

The effects of FUra on ribosomal RNA have been investigated for over 30 years. Early work showed that FUra interferes with the maturation and metabolism of the ribosome (Gros et al, 1962; Hignett, 1966; Takimoto et al., 1986). But overall, little information is known concerning the effects of FUra on rRNA structure and function. One sketchy study showed that the 23S RNA in the 50S subunit of bacterial ribosomes was more susceptible to ribonuclease degradation in FUra-treated cells, thereby altering the physical properties of the particles (Hahn and Mandel, 1971). The ratio of RNA to protein was greatly reduced in the 70S ribosomal fraction of FUra-treated cells, but was increased in the soluble fraction of the cells. This may be interpreted as a deficiency in the rRNA or as an excess of protein in the 70S ribosomal fraction of cells treated with the drug.

Incorporation of FUra into the 45S pre-rRNA, the precursor to the mature RNA molecules found in mammalian ribosomes, disrupts the secondary structure of the molecule and leads to an inhibition of rRNA processing (Weiss et al., 1974, Ghoshal et. al., 1994). The nuclear RNA (45S) is a precursor for the 28S, 18S, and 5.8S rRNAs. After methylation and conversion to a 41S molecule, the precursor is cleaved to 32S and 20S rRNAs. These molecules are further processed and assembled into 40S and 60S ribosomal subunits (eukaryotic cells) (Figure 11). Recent work has shown that maturation of FUra-containing rRNA is severely inhibited between the 45S and 32S stages of eukaryotic ribosome assembly pathway. The nucleotide analogue also inhibited the production of mature 18S and 28S rRNAs (Wilkinson et. al., 1973;

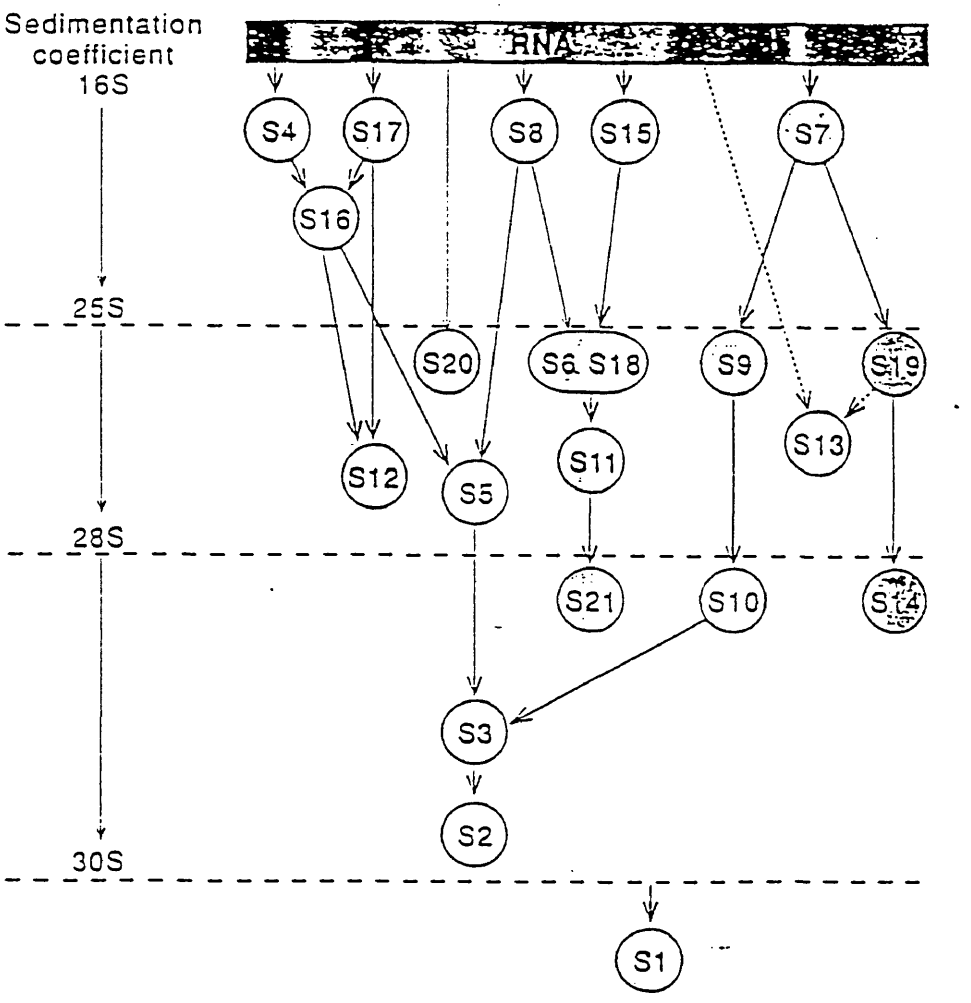


Figure 9. Assembly map of the 30S ribosomal subunit. Of the 21 proteins present in the subunit only 5 are directly associated to the rRNA. Assembly of the other proteins occurs only after these initial proteins have bound to the rRNA. These proteins make contact with the ribosomal RNA and the initially bound proteins (Darnell *et. al.*, 1990).

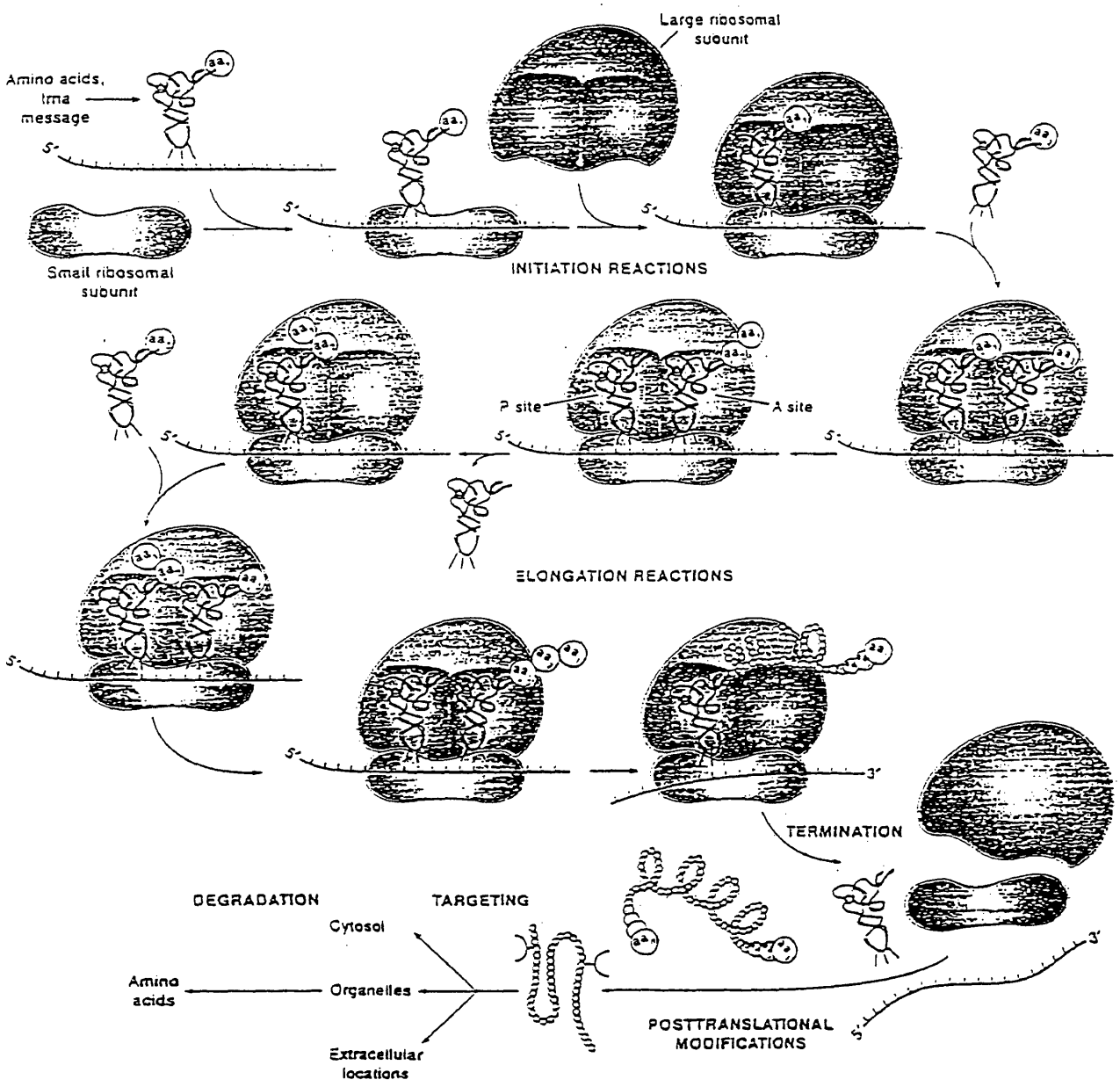


Figure 10. (Zubay, 3rd Ed.)

Figure 10. The three stages of translation in prokaryotic and eukaryotic cells. a). Initiation: This shows the assembly of the ribosomal subunits (30S and 50S) along with an initiation factor and a molecule of GTP to make the 70S complex. The complex hops on the mRNA transcript to find the start site of initiation, i.e. the AUG start codon. Once the first tRNA molecule carrying the first amino acid becomes bound to the ribosome at the peptidyl-tRNA site (P-site), translation is ready to begin. Elongation: As the ribosome moves along the message, the polypeptide chain elongates with a new tRNA molecule bringing in a new amino acid at the A site. The peptidy-tRNA is now at the A site of the ribosome. The process is catalyzed by a protein complex (elongation factors). The empty tRNA molecule exits out of the ribosome as the ribosome moves along the message. Termination: The ribosome comes to a stop codon indicating the end of translation. This occurs with the aid of termination factors, and GTP which causes hydrolysis of the peptydyl-tRNA and the dissociation of the ribosomal subunits. (Darnell *et. al.*, 1990).

Cohen and Glazer, 1985). The recognition sites involved in processing appear to be altered, suggesting that incorporation of FUra into preribosomal RNA is responsible for an altered processing mechanism (Herrick and Kufe, 1984). Kanamaru et. al. (1986) disputed these results by showing that the inhibition of the processing of the pre-rRNA is not due to the accumulation or incorporation of FUra-particles. In Kanamaru's study, the 18S rRNA was intact at high concentrations of FUra. Cells incubated in a 10^{-5} M FUra for 24 hours displayed inhibited processing of 18S rRNA as well as processing to lower amounts of 32S preribosomal RNA and 28S rRNA. Assays of cells labeled with ^{14}C-UR (labeled uridine) following a 24 hour exposure to ^{3}H-FUra gave no processed 28S rRNA, instead newly synthesized RNA accumulated in the regions of 45S and 32S preribosomal rRNA. The conclusion of the study was that the processing of pre-rRNA to 28S RNA is due to some suggested unknown mechanism, and not to the accumulation of FUra-particles. However, the mechanism to explain FUra cytotoxicity is still not fully understood.

In that same year, Takimoto et al., (1986) showed that different precursors of FUTP do not produce the same type of incorporation into RNA. Two precursors of FUra were used to determine FUra distribution into RNA under cytotoxic and non-cytotoxic conditions. The results showed that high doses of FdUrd on a time dependent basis gave very little RNA mediated cytotoxicity. On the other hand, high levels of cytoxicity and incorporation into RNAs resulted when small concentrations of FUrd were used. This was explained by the fact that 5'-deoxyfluorouridine (5'-dFUrd) incorporation into nuclear RNA is equally distributed between the nucleoplasmic RNA and nucleolar RNA giving non-toxic

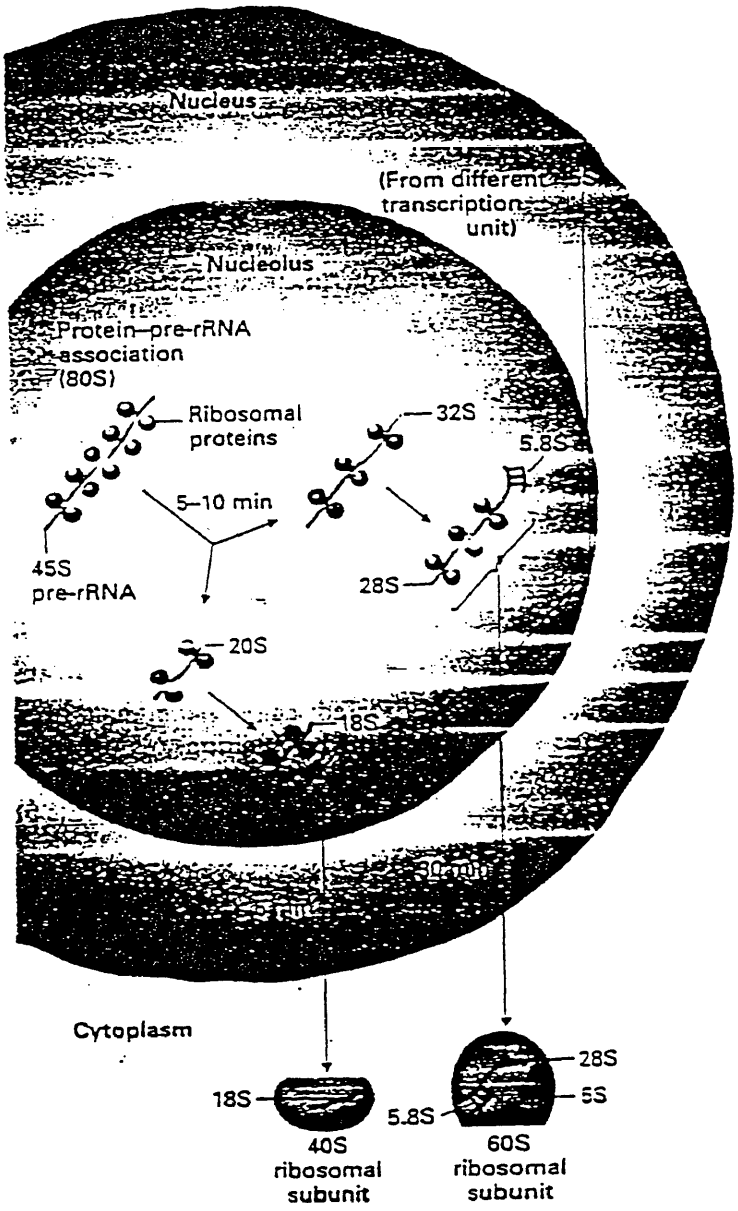


Figure 11. Processing of mammalian 45S pre-rRNA into ribosomal subunits. Processing begins in the nucleolus. The 45S pre-rRNA is processed into a 32S and 20S rRNA before they are transported to the cytoplasm where they get synthesized into the 40S and 60S ribosomal subunits (Darnell *et. al.*, 1990).

doses of FUra in the cell. In contrast, FUra incorporation from a FUrd precursor was high because it targeted nucleolar RNA. In addition, the rapid conversion of FUrd to FUTP explains the high incorporation of FUra in newly synthesized RNA and induces physiological changes that prevent these RNA molecules from reaching maturity.

Studies done within the last five years indicate that there are still some unanswered questions about the cytotoxicity of FUra (Takimoto et. al., 1993). In spite of the amount of work done, very little information on FUra effects on the structure and function of rRNA exists. To provide additional information on the effects of FUra on the metabolism of cells, this research project has addressed some specific structural and functional effects of FUra on rRNA in the ribosomal system of *E. coli.*

EXPERIMENTAL DESIGN

The focus of this research project was to identify and observe the effects of FUra on protein translation, specifically the effects of FUra on the structure and function of the ribosome. Initially, growth rate experiments using a range of concentrations of FUra were used to define those FUra concentrations which inhibit cell growth or significantly retard growth. Concentrations of FUra between 1ug/ml and 200 ug/ml were utilized in this study. The growth rates of *E. coli* cells treated with these range of FUra concentrations were done to observe the effects of the drug during cell growth. Using FUra concentrations between 1 ug/ml and 50 ug/ml, whole ribosomes and polyribosomes were prepared and the absorbancy profiles of each sample was produced. RNA

derived from these FUra-treated cells was also analyzed on a polyacrylamide gel for evidence of degradation due to the drug. The effects of FUra on ribosomal proteins were studied by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

The structure and conformation of ribosomes from FUra-treated cells was characterized on composite gels. An incorporation assay was also carried out to determine if the drug was being incorporated into the cells and also to identify the specific portion of the ribosomal pool most affected by FUra. The results of this study expand on previous findings concerning the effects of FUra on the structure and function of the ribosome. The results also support the notion that the ability of FUra to effectively reduce growth in tumor cells depends upon its ability to alter the functional capabilities of the ribosome.

MATERIALS AND METHODS

GROWTH RATE EXPERIMENTS

To observe the effects of 5-fluorouracil (FUra) on the growth of *E. coli* cells, cultures were monitored by measuring turbidity of media over time. An aliquot containing 500 ul of an overnight culture of *E. coli* DH1 strain was used to inoculate 50 ml of Luria-Bertani (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L H_2O) containing the following FUra drug concentrations; 0 ug/ml, 1 ug/ml, 10 ug/ml, 25 ug/ml and 50 ug/ml. The drug was added from a stock solution. The cultures were allowed to grow at 37^{o}C in a shaking water bath for about eight hours. Using a Klett-Sommerson colorimeter, the turbidity of the growing cultures was monitored every 30 min.

To determine the effects of FUra on cells that were already growing, another growth rate experiment was carried out using 200 ug/ml of FUra. In one experiment, 200 ug/ml of FUra was added to the media before the cells were inoculated (an experiment similar to that described above). In a parallel experiment, 200 ug/ml of FUra was added to a culture that contained cells that had been growing for about 3 hours. Both cultures were left to grow for about six-and-a-half hours. Turbidity readings were monitored as in the previous experiment (every 30 min) using the Klett-Sommerson colorimeter. The results of the growth experiments were used to determine the level of FUra to be used in subsequent experiments.

PREPARATION OF RIBOSOMES

The preparation of ribosomes was carried out according to the protocol provided by Tapprich & Dahlberg (1990). It involved growing *E. coli* cells in 500 ml of LB nutrient medium until they reach mid-logarithmic growth phase ($A_{600\ nm}$ = 0.6) after which the cells were harvested. Ribosomes were isolated from cells grown in 9 different FUra concentrations; 10 ug/ml, 20 ug/ml, 30 ug/ml, 40 ug/ml, 42.5 ug/ml, 45 ug/ml, 47.5 ug/ml, and 50 ug/ml. In each case, the drug was added before the fresh cultures were inoculated with an overnight culture. The cells were harvested by first cooling them in two GSA centrifuge tubes (two tubes per 500 ml culture) on ice for 5 min and then centrifuging in a GSA rotor at 4^{o}C for 10 min at 5000 rpm. The medium was poured off and the pellet was washed by resuspending it in 7.5 ml of Buffer A (25 mM Tris-HCL / pH 7.6, 10 mM $MgCl_2$, and 150 mM KCl, and 1 mM DTT). The washed samples were then transferred into prechilled SS34 tubes and centrifuged again at 4^{o}C for 10 min at 5000 rpm. The supernatant was poured off and the pelleted samples were kept overnight in a -70^{o}C freezer.

To extract ribosomes, the frozen pellets were scraped into a precooled mortar on ice. An aliquot containing 3.4 g of baked alumina was added and the cells were ground with a prechilled pestle until the cells formed a slightly thick paste. As the cells were broken open, the paste became smoother in consistency. An aliquot containing 50 units of RNase-free DNase I (Worthington Biochemicals) was added to the paste and then 6 ml of Buffer A was added to the paste gradually so that it could be poured into a prechilled SS34 tube. An

additional 6.5 ml of Buffer A was added to the samples to have a total volume of 12.5 ml. The cells were then centrifuged in the SS34 rotor at 4°C for 10 min at 12,000 rpm to remove the alumina and other large cellular debris from the cell lysate. The supernatant was placed into a clean, prechilled SS34 tube and centrifuged again to remove any more cellular debris at 4°C for 1 hour at 16,000 rpm. Following centrifugation, the supernatant was transferred into prechilled Ti-70 tubes, filled to the top with Buffer A, and centrifuged in a Beckman Ti-70 rotor for 3 hours at 60,000 rpm (4°C), the supernatant was removed and the ribosomal pellet was resuspended in 10 ml of Buffer B (10 mM Tris-HCl / pH 7.6, 15 mM $MgCl_2$ and 500 mM NH_4Cl in 1 mM DTT) and left on a tilt table at 4°C overnight.

The resuspended pellets were transferred into prechilled SS34 tubes and centrifuged for 1hour at 16,000 rpm in a Sorvall centrifuge at 4°C after which the supernatant was decanted into prechilled Ti-70 tubes. These tubes were again filled to the top, this time, with Buffer B, and centrifuged in a Ti-70 Beckman rotor for 3 hours at 60,000 rpm also at 4°C. The supernatant was removed again and the pellet was resuspended in 500 ul of TC70S buffer (20 mM Tri-HCl / pH 7.6, 6 mM $MgCl_2$, and 60 mM KCl). As before, the samples were kept on a tilt table overnight in the cold room. The dissolved pellets were centrifuged in a microcentrifuge in the cold room for 10 min at 15,000 rpm to separate the ribosomes from insoluble cellular materials. The dissolved pellet (supernatant) was put into clean eppendorf tubes and ribosomal concentrations

were measured using a spectrophotometer.

An absorbance reading at 260 nm of a 1:250 diluted sample was made using an extinction coefficient of 14.5. Based on the calculated concentrations of the extracted ribosomes, 6 mg of each sample was loaded onto a previously prepared (thawed) 38 ml sucrose gradient (22% sucrose in Tight Couple70S buffer (TC70S) containing 1 mM DTT). The loaded gradients were centrifuged at 4^{o}C for 18 hours at 20,000 rpm in a Beckman SW28 rotor. Acceleration-decceleration parameters were set so that the rotor changed speeds gradually at low speeds; 3 min between the rates of 0 and 500 rpm. The resulting gradients were pumped through an Isco Apparatus UA-5 Absorbance/Fluorescence detector at a pump speed of 70, a sensitivity of 1 and a chart speed of 60 cm/hr. Each fraction of ribosomal 30S, 50S and 70S particles was collected in RNase-free SS34 tubes. A profile for each subunit (30S, 50S, and 70S) was generated as each fraction was collected. The 30S, 50S and 70S fractions were then put into Ti-70 tubes, filled to the top with TC70S buffer and centrifuged again in the Beckman Ti-70 rotor for another 18 hours at 40,000 rpm at 4^{o}C. The pelleted ribosomal fractions were then resuspended in 500 ul of TC70S buffer. The 70S fractions were kept in the freezer (-70^{o}C) for further studies. The entire procedure was done at 4^{o}C and in RNase-free conditions. RNase-free conditions were accomplished by using purified buffers made with sterilized water and filtered before using, glass apparatus, mortars, pestle and spatulas were baked to inactivate RNase enzymes. The plastic tubes used were also exclusively for RNA work.

POLYACRYLAMIDE GEL ELECTROPHORESIS

To observe any degradative effects of the drug on the RNA derived from the 30S and 50S ribosomal subunits, the ribosomal RNA was isolated from previously prepared subunits and was analyzed by polyacrylamide gel electrophoresis. The purification of rRNA involved phenol extraction. First, an equal volume of a buffer-equilibrated phenol was added to the ribosomal fractions, vortexed for 15 sec, and then centrifuged for 2 min in a microfuge at 15,000 rpm at room temperature to separate the phases. The upper aqueous layer was retained and treated with an equal volume of buffer-equilibrated phenol-chloroform solution (phenol : chloroform: isoamyl alcohol, 25:24:1). The samples were vortexed again for 15 seconds and centrifuged at the same speed for another 2 min. The upper, aqueous phase was retained and precipitated with 1/10 volume of a 3 M sodium acetate pH 5.2 and 2.5 volumes of cold 95% ethanol, mixed well and kept in the -70^{o}C freezer for about 20 min. The samples were centrifuged at 15,000 rpm for 10 min at 4^{o}C to pellet the RNA. The supernatant was discarded and 0.5 volumes of cold 70% ethanol was added before it was centrifuged for 5 min at 15,000 rpm at 4^{o}C. The supernatant was discarded carefully and the RNA pellet was dried in the speed-vac for 3 min before it was resuspended in 50 ul of Tris-EDTA (TE contained 10 mM Tris / pH 7.6, 1 mM EDTA). Absorbance values at 260 nm were determined and the concentration of each sample was calculated using an extinction factor of 25. An aliquot containing 10 ug of each sample was loaded onto a 3.75% polyacrylamide gel.

The polyacrylamide gel contained 8 M urea (26.4 g), 3.75% acrylamide (19:1 acrylamide : bisacrylamide), and 1 X TBE (0.09 M Tris-Borate 0.002 M EDTA). The solution was stirred on a hot plate to dissolve and mix the components after which the volume was brought to 55 ml. Polymerization was initiated by adding 150 ul of TEMED and 200 ul of 10% APS (Ammonium persulfate) to the mixture. The mixture was swirled quickly and then poured in between two glass plates separated by 1.5 mm spacers. A 10-tooth comb was inserted between the plates to make the wells before the gel polymerized. The samples were prepared by placing 10 ug of each sample into a 0.6 ml eppendorf tube along with an equal volume of 8 M urea and 1 ul of 2.25% bromophenol blue. The samples were electrophoresed for approximately 6 hours at 13 mA in 1X TBE buffer. Following electrophoresis, the gel was stained overnight in 200 ml of a solution containing 0.2% methylene blue, 0.2 M sodium acetate, 0.2 N acetic acid. The gel was destained with deionized water and then dried.

SDS-PAGE ELECTROPHORESIS

To observe the profile of ribosomal proteins asociated with the ribosomes from FUra-treated cells, approximately 30 ug of ribosomal protein preparation was loaded on a 15% SDS separating gel (20 ml of 30% acrylamide, 8 ml of 1.875 M Tris-HCl pH 8.8, 400 ul of 0.2 M EDTA, 20 ul of TEMED, and 400 ul of 10% APS in a total volume of 40 ml). The stacking gel was 5% acrylamide (1.67 ml of 30% acrylamide, 1.25 ml of 1 M Tris-HCl pH 6.8, 100 ul of 0.2 M EDTA, 100 ul of 10% APS, and 5 ul of TEMED in a total volume of 10 ml).

The separating gel was poured between two horizontal layered with water and then left to polymerize for about 20 min after which the stacking gel was prepared and poured on top of the separating gel (after removing the layer of water from the top of the gel). The comb was inserted into the stacking gel to make wells and left to polymerize again for another 30 - 40 min after which, the comb was removed and the wells were flushed with the top running buffer to remove any particles that might interfere with the smooth migration of the protein samples. The top chamber was then filled with a full concentration of the running buffer (0.025 M Tris, 0.19 M glycine, 0.003 M SDS in a total volume of 4 L at pH 8.3). The bottom electrophoresis chamber contained about 3 L of a 1:2 dilution of the same running buffer. To prepare the samples, an equal amount of a 2X solubilization buffer (2 ml of 10% SDS, 400 ul of 2-mercaptoethanol, 1 ml of glycerol, 625 ul of 1 M Tris pH 6.8 and H_2O to make a total volume of 5 ml with bromophenol blue to give it color) was added to each sample. The solubilized samples were boiled for 5-10 min at 100^{o}C and then loaded onto the gel. The gel ran for 2.5 - 3 hours at 50 mA before it was stained overnight in 2% coomassie blue dye (0.2 g coomassie, 45 ml methanol, 45 ml water, 10 ml glacial acetic acid). The gel was placed in a destaining solution (Per liter: 7% glacial acetic acid, 25% isopropyl alcohol in distilled water) overnight.

COMPOSITE GEL ELECTROPHORESIS

To characterize the overall conformation of FUra-treated ribosomes, composite gels were prepared. The procedure for this type of ribosomal

separation was done according to Dahlberg and Grabowski (1990). These gels give very good resolution of large complex macromolecules and allow separation based on size and shape. The gels were made up of a mixture of agarose and acrylamide. A 2.25%- 0.5% acrylamide - agarose gel (0.8 g agarose in 127 ml of ddH_2O, 9 ml of 40% acrylamide solution, 4 ml of 1 M Tris-HCl pH 7.6, 1.6 ml of 3 M KCl, 1.6 ml of 1 M $MgCl_2$, 500 ul of 10% APS, and 100 ul of TEMED) was prepared such that the entire gel mixture was kept at about 43°C. Before preparing the gel, the cooling system was turned on so as to bring down the temperature to about 20°C. The gel was prepared by dissolving the agarose in 126 ml of ddH_2O. This was done by heating gently on low heat. The flask and its contents were cooled down to approximately 60°C by keeping it under a running faucet of cool water. The volume was readjusted by adding ddH_2O before adding 25 mM Tris-HCl, 10 mM $MgCl_2$, and 30 mM KCl. The total volume of the gel mix was about 160 ml. The flask and its contents were then cooled down to about 43°C.

Meanwhile, once the circulating cooling system was adjusted and brought to 20°C, it was left to circulate around the wires of the gel slab for about 10 min. As soon as the gel mix cooled down to ~ 43°C, 1.6% APS was added. The flask was swirled very quickly and the gel mix was poured into the gel mould in the cooled unit. The comb was inserted to form the wells and a frozen tube of ethylene glycol was placed in front of the comb to aid in cooling the gel areas that could not be cooled by the cooling coils. The gel was poured at

20°C to allow the agarose to set before the acrylamide and also to form a coat around the gel slab, thereby giving the gel stability. The gel was kept in the horizontal position for an hour to allow the entire gel to polymerize. A precooled 1X TMK buffer - the running buffer (25 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, and 30 mM KCl) was poured into the gel chamber (now in the vertical position). Pre-electrophoreses was done by running the gel for 1 hour at 50 V while reducing the temperature of the coolant to 4°C.

Ribosome samples were prepared by mixing 20 ug of the previously prepared ribosomal fractions in 30ul of TC70S buffer (the buffer in which the ribosomal samples were dissolved). An equal volume of a 50°C, 0.5% agarose in the same TKM buffer concentration was added to the sample. Before loading the samples, the running buffer in the gel chamber was drained. The wells were blotted dry using small pieces of filter paper before the samples were loaded onto the empty, dry well. The gel was run at a constant voltage of 100 V at 4°C for about 6 hours recirculating the buffer constantly and replacing the running buffer with fresh, cold running buffer every 2 hours. After 6 hours, the gel was stained in 2% methylene blue overnight and then destained in deionized water.

POLYRIBOSOME PREPARATION

The effects of FUra on translating ribosomes was also observed in the polyribosome profiles derived from sucrose gradients of *E. coli* cell lysates. These were prepared according to the protocol by Vila et. al. (1994). An

overnight culture of *E. coli* cells was used to inoculate 50 ml of fresh LB nutrient media containing the following 5-FU concentrations; 20 ug/ml, 30 ug/ml, 40 ug/ml, 47.5 ug/ml and 50 ug/ml. The cultures were left to grow until they reached mid-logarithmic growth phase ($A_{600\ nm}$ = 0.6) before the cultures were cooled quickly in an ice bath by swirling the culture in the bath for 5 min. The cells were pelleted by centrifugation at 4^{o}C at 6,000 rpm for 5 min in a Sorvall SS34 rotor. The supernatant was removed and the pellet was washed in 1 ml of TKM buffer (25 mM Tris pH 7.6, 25 mM $MgCl_2$, 60 mM KCl in 20% sucrose and 150 ul/ml lysozyme) and then transfered into an eppendorf tube before it was pelleted again for 10 min at maximum speed in a microcentrifuge at 4^{o}C. After removing the supernatant, 80 ul of the above TKM buffer was added to the pellet and resuspended with the aid of a sterile toothpick. Five cycles of freezing and thawing was carried out to weaken the cell walls of these cells and then 300 ul TKM buffer (without sucrose or lysozyme), 60 ul of a neutral lysis solution Brij 58 (5% w/v in 10 mM Tris pH 8.0), 100 units of RNase-free DNase I (Worthington biochemicals), 100 ul deoxycholic acid reagent (1% in 10 mM Tris pH 8.0) and 25 ul $MgSO_4$ (0.1 M) was added to each sample. The samples were left to on ice for 15 min and then centrifuged in the microcentrifuge for 10 min at maximum speed in the cold room. The supernatant (~610 ul) containing the ribosomes and polysomes were transfered into new, clean eppendorf tubes and the concentration of the polysomes and ribosomes was determined spectrophotometrically using a 1:100 dilution of the samples at an absorbance of 260 nm and an extinction coefficient of 14.5. The lysate was

brought to 1ml with the TKM solution and then loaded onto a 38 ml 5-30 % sucrose gradient in TKM buffer (25 mM Tris-HCl pH 7.6, 15 mM MgCl2, 60 mM KCl) and centrifuged at 26,000 rpm at 4^{o}C for 6 hours in a Beckman SW28 rotor with the acceleration - decceleration parameters set for the rotor to change its speed gradually at low speeds (3 minutes between the rates of 0 and 500 rpm).

The resulting gradients were pumped through an Isco Apparatus UA-5 Absorbance/Fluorescence detector at a pump speed of 60, a chart speed of 70 cm/hour at a sensitivity of 1 all at 260 nm. The separate fractions (30S, 50S, and 70S and polyribosomes were collected and kept in a -20^{o}C freezer with two times the volume of 95% ethanol.

INCORPORATION STUDIES

The amount of 5-fluorouracil incorporated into the ribosomes was measured in a separate study using tritiated-FUra (^{3}H-5FU). Basically, this experiment was similar to the procedure for polyribosome preparation previously described. In this case, five flasks containing 50 ml of LB nutrient medium were inoculated with 500 ul of an overnight culture and the cells were allowed to grow at 37^{o}C for 100 min. After that, the cells were labeled with ^{3}H-FUra (6 uCi / 50 ug/ml). The flasks were placed back in the water bath to allow the cells to continue the growth process. The fifth flask contained no FUra (control) and was kept in the waterbath until the cells reached an A_{600} value of 0.6 (mid-logarithmic growth phase) and then harvested. The other four flasks were harvested at different time intervals. Before harvesting, 100 ul of the FUra-

treated culture was put in an eppendorf tube and kept in the refrigerator. Cells in Flask #1 were harvested after 60 min of incubation of the tritiated FUra. Cells in Flask #2 were harvested 90 min after incubation, cells in Flask #3 were harvested 30 min (120 min) later and cells in Flask #4 were harvested another 30 min later (150 min). The rest of the polyribosome preparation was carried out according to Vila et. al, (1994) in the protocol described in the previous section.

Once the lysates were loaded unto a 38 ml 5-30% sucrose gradient in TKM buffer, they were centrifuged at 26,000 rpm at 4^{o}C for 6 hours in the Beckman SW28 rotor with the acceleration - deceleration parameters set as in the previous experiment (rotor set to change its speed gradually 3 minutes between the rates of 0 and 500 rpm).

The gradients were pumped through the Isco Apparatus UA-5 Absorbance/Fluorescence detector pump using a chart speed of 60 cm/hr, a pump speed of 70 and a sensitivity of 1 at an absorbance of 260 nm. Fractions of 20 drops were collected in eppendorf tubes for absorbancy and radioactivity assays. Aliquots containing 100 ul of each of these fractions were put on a filter paper, dried in an incubator set at 50^{o}C for approximately 10 mins. Each filter was placed in liquid scintillation vials and 4 ml of scintillation fluid (cocktail) was added. Each sample was counted for 4 min. The same was done to the 100 ul of the cell lysates removed before harvesting and before loading onto the gradient.

5-fluorouracil and tritiated-FUra were gifts from Dr. William Gmeiner of the

University of Nebraska Medical Center in Omaha.

RESULTS

The object of this study was to investigate the effects of 5-fluorouracil on the structure and function of the ribosome. FUra is a drug that is known to be effective in retarding the growth processes of certain tumor cells. The results of this study has contributed to the body of work characterizing the effects of FUra at the molecular level. The study has also shown that there are still many unanswered questions about FUra.

DOES FUra AFFECT THE GROWTH RATE GROWTH ?

The effects of 5-Fluorouracil on the growth of *E. coli* cells were observed for growth in rich media at 37^{o}C, which allow the translational apparatus of the cell to function at maximal rate and efficiency. The data for the growing of cells in several concentrations of FUra are shown in Figure 12. Experimental conditions were employed using FUra concentrations varying from 1ug/ml - 50 ug/ml. Cells grown in 1ug/ml of FUra (5FU1) showed only a slightly decreased growth rate compared with control cells (5FU0). A ten-fold increase in the drug concentration (5FU10) showed a remarkable decrease in the growth rate indicating a decreased rate of division. Cells grown in 25 ug/ml of FUra (5FU25) and 50 ug/ml of FUra (5FU50) showed only slight growth 8 hours after inoculation. Very little growth in these cultures was apparent throughout the experiment. Thus, they did not divide enough times to show significant growth characteristics.

A similar experiment was performed to assess the effects of FUra on cells

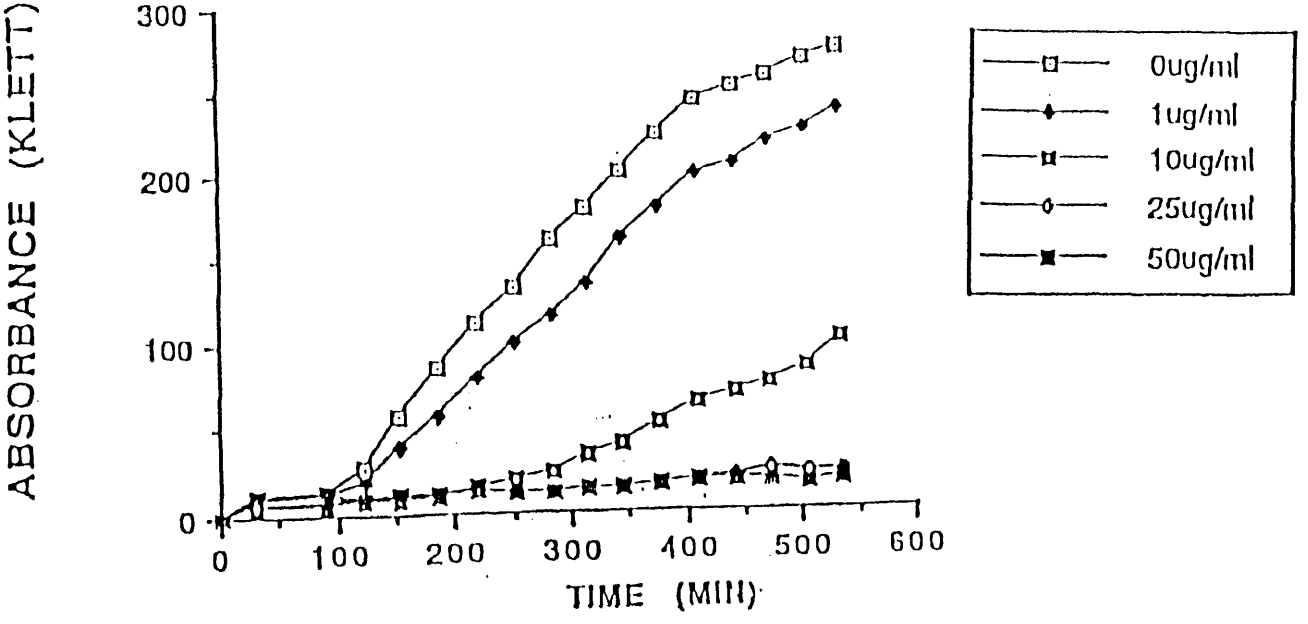


Figure 12. Growth of *E. coli* cells following exposure to FUra. 500ul of an overnight culture was used to inoculate 50 ml LB nutrient medium with the shown concentrations of FUra at 37^{o}C.

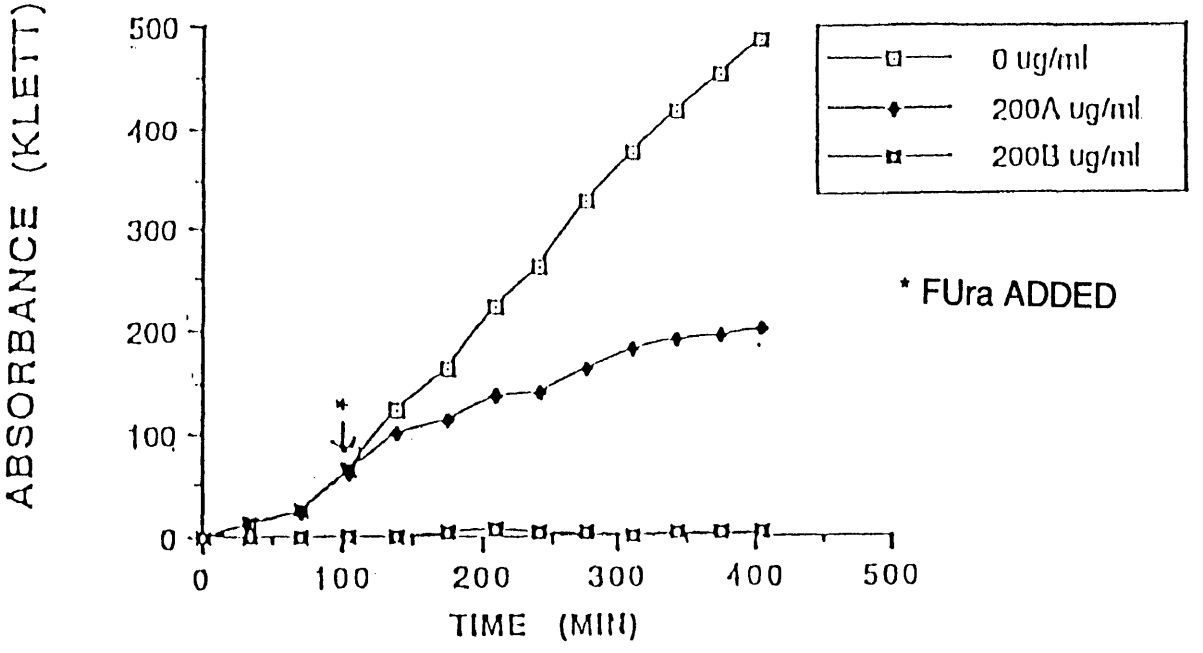


Figure 13. Growth of *E. coli* cells after the addition of FUra during growth. The filled diamonds show the rate of growth when cells were inoculated to the media with 200 ug/ml FUra at 0 min. The filled boxes show the effects of the same concentration of FUra was added after 100 min after inoculation as indicated by the asterix. Both experiments were carried out at 37^{O}C at the same time as described in Methods.

that were already growing. Here, cells were grown for 100 min before 200 ug/ml FUra was added (5FU200A). These cells showed normal growth characteristics until the addition of FUra when they exhibited a markedly decreased growth rate (Figure 13). A control experiment done with 200 ug/ml FUra present at the time of inoculation (5FU200B) gave a pattern of growth similar to that seen previously in Figure 12 for the cells grown in 25 ug/ml and 50 ug/ml.

Both experiments showed that cells grown in the presence of FUra had a decreased rate of growth. No differences were observed in cells grown in 5FU25, 5FU50 or 5FU200(B) indicating that there is a threshold value at which the drug exerts its maximum potential on the growth of the cell.

DOES FUra AFFECT THE FORMATION OF FUNCTIONAL RIBOSOMES ?

To analyze the effects of FUra on the formation of functional ribosomes, isolated ribosomes from cells grown in LB medium containing 9 different FUra concentrations were prepared and analyzed. Cells were grown to an A_{600} of 0.6 at 37^{o}C then ribosomes were prepared individually from cultures containing 1, 10, 20, 30, 40, 42.5, 45, 47.5 and 50 ug/ml concentrations of FUra. The drug was added to the medium before inoculation. These ribosomes were treated in a high concentration (0.5 M) of NH_4Cl which removes cellular factors from the ribosomes. The ribosomal preparations were loaded on a sucrose gradient to separate ribosomal particles. Cells grown in FUra concentrations between 30 and 50 ug/ml of FUra took about 4-5 hours longer to reach A_{600} of 0.6. The

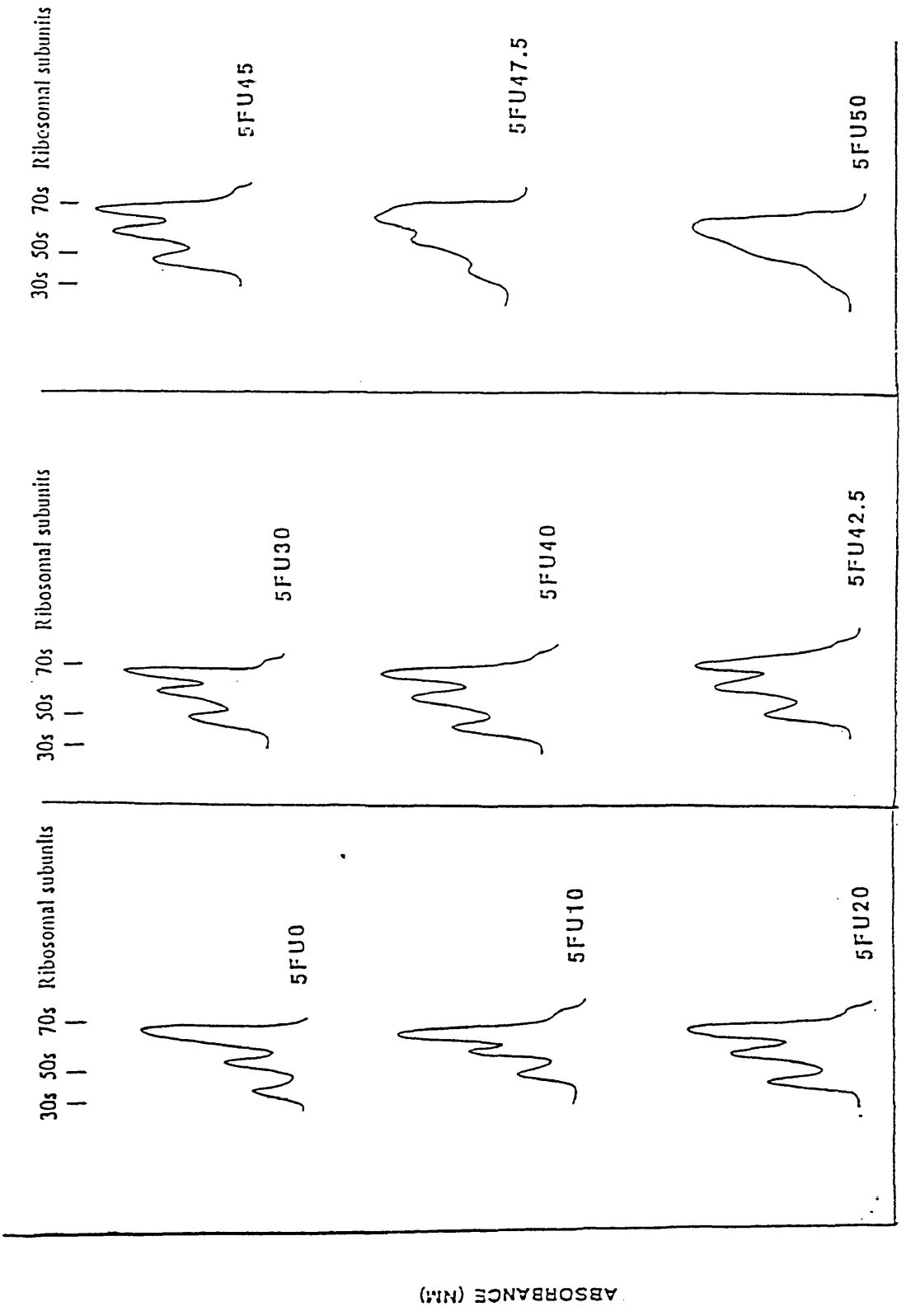

30s 50s 70s Ribosomal subunits
5FU0
5FU10
5FU20
30s 50s 70s Ribosomal subunits
5FU30
5FU40
5FU42.5
30s 50s 70s Ribosomal subunits
5FU45
5FU47.5
5FU50
ABSORBANCE (NM)
FRACTION NUMBER

Figure 14.

Figure 14. Sucrose density gradient profiles of cell lysates containing FUra concentrations between 0 ug/ml (5FU0) and 50 ug/ml (5FU50). Cells were collected from a 500 ml culture grown to an A_{600} = 0.6 in LB medium and processed as described in Materials and Methods according to Tapprich & Dahlberg (1990). Ribosomal pellets were dissolved in 1 ml TC70S buffer. 6 mg of this ribosomal preparation was loaded onto a 5-30% sucrose gradient (w/v) under the same buffered conditions and centrifuged for 18 hr at 20,000 rpm in a Beckman SW 28 rotor. The 30S, 50S and 70S subunits were collected separately as described in Methods.

absorbancy profiles were generated for each sample and the ribosomal fractions for each sample was collected in separate pools. The data in Figure 14 shows that in 5FU0, the distribution pattern of fractions is characterized by a large peak corresponding to associated ribosomal particles (70S) and the two smaller peaks corresponding to the free, unassociated subunits (30S and 50S). The distribution patterns of FUra-treated cells varied from that produced by the control. The 70S peak is smaller with increasing FUra concentrations (5FU10 - 45) and the 30S and 50S subunit peaks are larger (Figure 14). Cells in 5FU47.5 and 5FU50 gave peculiar absorbance profiles with the 30S and 50S peaks becoming a slight shoulder on the 70S peak and then becoming merged into one peak as seen in the 5FU50 sample.

The results indicate that with increasing FUra concentrations, free ribosomal subunits increase in the ribosomal pool while associated ribosomal complexes decrease in number. Also, the definite broadening of peaks suggests that the conformation of the ribosome is altered and unfolded particles are nonspecifically associating together. Since the functional ribosomes form discrete 70S peaks, increasing concentrations of FUra lead fewer functional ribosomes.

DOES FUra AFFECT THE TRANSLATIONAL MACHINERY *IN VIVO* ?

To analyze the effects of FUra on the translational machinery (the 70S complex and polyribosomes) in *E. coli* cells, ribosomal fractions from cultures containing four of the nine FUra concentrations: 20 ug/ml, 30 ug/ml, 47.5 ug/ml and 50 ug/ml were produced. Cells were grown in LB medium containing one

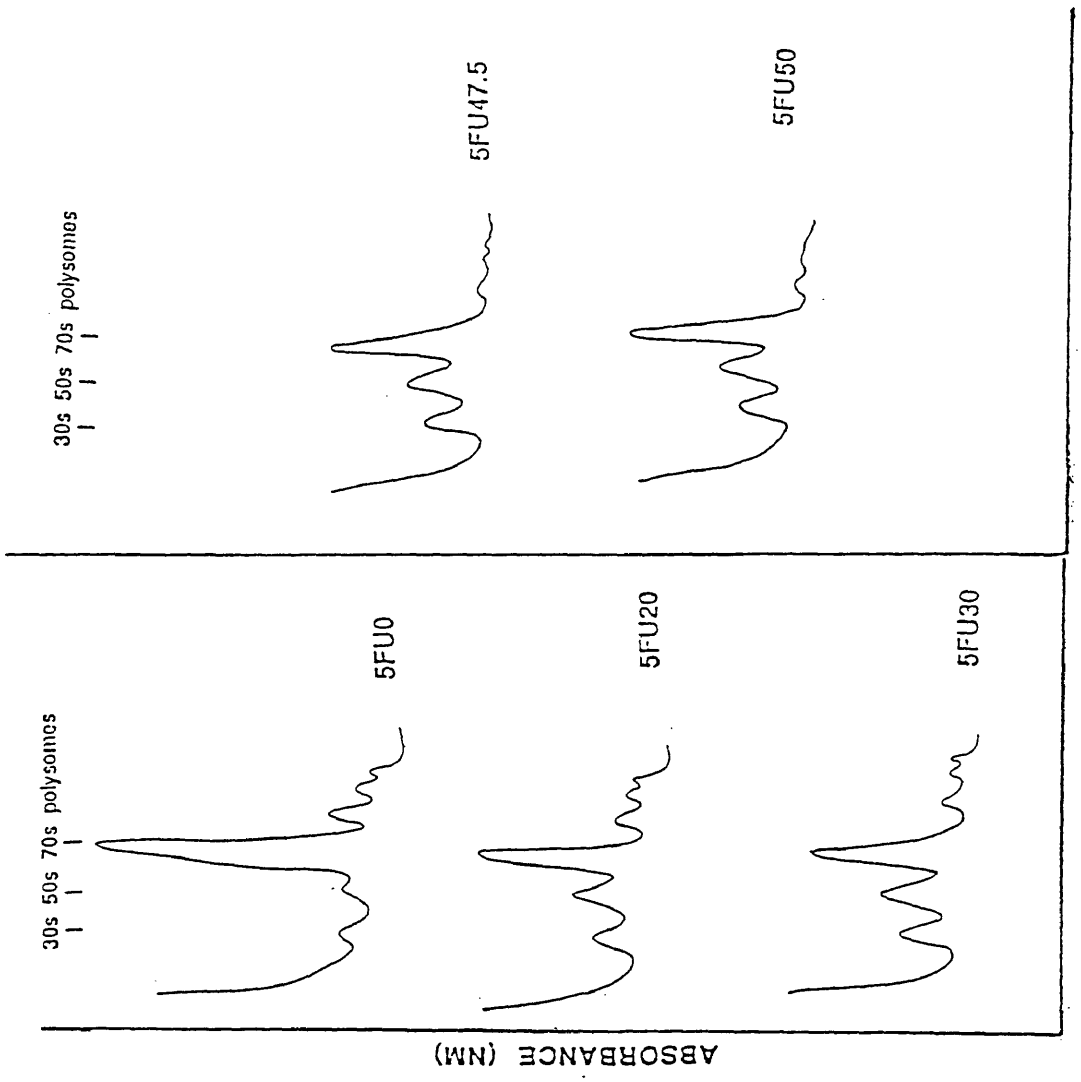

30s 50s 70s polysomes
5FU0
5FU20
5FU30
30s 50s 70s polysomes
5FU47.5
5FU50
ABSORBANCE (NM)
FRACTION
FRACTION

Figure 15.

Figure 15. Sucrose density gradient profiles of cell lysates isolated from FUra-treated cells. Cells were grown in 50 ml LB cultures containing a range of FUra concentrations between 0 ug/ml (5FU0) anf 50 ug/ml (5FU50). Cell lysates were collected as described in Methods according to procedures by Vila *et. al.*, (1994). The pellet was resuspended in 1 ml TKM buffer and loaded onto a 5 -30% (w/v) sucrose gradient under the same buffered conditions. Samples were centrifiuged for 6 hours at 26,000 rpm at 4^{o}C in a Beckman SW28 rotor. The polyribosome fractions were collected as described in Methods.

of the four FUra concentrations. Once the cells reached an A_{600} of 0.6 at 37°C they were immediately chilled in an ice bath with constant swirling to quickly cool the cells. The cells were harvested and treated in a buffer which had a higher magnesium concentration than in the previous experiment (25 mM vs 6 mM for lysis and 10 mM vs 6 mM for the gradient). By analyzing whole cell lysates, this experiment also assayed ribosomes in the presence of cellular factors, a condition which differs markedly from NH_4Cl salt-washed ribosomes. The absorbance profiles generated for each of these samples are shown in Figure 15. With increasing drug concentration, there was a decrease in the fraction of associated ribosomal subunits forming the 70S complex. All FUra-treated samples had larger 30S and 50S peaks compared to the control sample, with the 70S fraction being significantly smaller than the control 70S peak. In addition, the profiles of samples treated with 20 ug/ml of FUra showed that there are polyribosomes present. The polyribosome peaks in this sample is similar to the control polyribosome peaks. However, the polyribosome peaks in 5FU30 were lower than in the control or 5FU20. The samples with the highest drug concentrations (5FU47.5 and 5FU50) gave profiles showing little or no polyribosomes present, larger peaks of free 30S and 50S subunits, and smaller 70S peaks. These profiles showed a definite effect of FUra on the translational machinery of *E. coli in vivo*.

DOES FUra LEAD TO DEGRADATION OF rRNA ?

The effects of FUra on RNA degradation was observed by running equal amounts of purified rRNA samples from previously isolated ribosomal fraction

on a 3.75% polyacrylamide gel. The normal break down of 23S rRNA can be seen in 5FU0 in Figure 16. The incorporation of FUra into the 23S rRNA of the 50S subunit showed that the breakdown products increased with increasing FUra concentrations. The 23S rRNA band is decreased as FUra concentrations increase while a well characterized breakdown product, the 13S rRNA, is increased. In 5FU47.5 and 5FU50, the 18S RNA band is further broken down to give two more distinct bands of break down products. The intensity of the 5S rRNA band varied slightly from sample to sample. This molecule can be used to normalize the amount of rRNA in each lane.

In the 16S rRNA, the effects of FUra are not as drammatic as seen in the 23S rRNA. In this case, the same break down products are seen in all samples (data not shown). The only difference observed is in the 5FU50 sample which shows a slower migrating band than any of the others. This reflects the presence of 23S rRNA contaminating the 16S prep. Note that this sample was derived from a gradient such as that shown in Figure 14. Thus, it was not a pure 30S fraction. As shown in Figure 14, the absorbance profile of this sample showed that all ribosomal particles migrated as one merged peak.

DOES FUra AFFECT RIBOSOME STRUCTURE ?

Composite gel electrophoresis permits the study of the ribosome structure by separation of molecules based on size and shape. Changes in electrophoretic mobility reflects conformational changes in the ribosome structure (Dahlberg and Grabowski, 1990). In this experiment, ribosomal lysates for 8 of the 9 previously prepared FUra-treated samples were loaded on

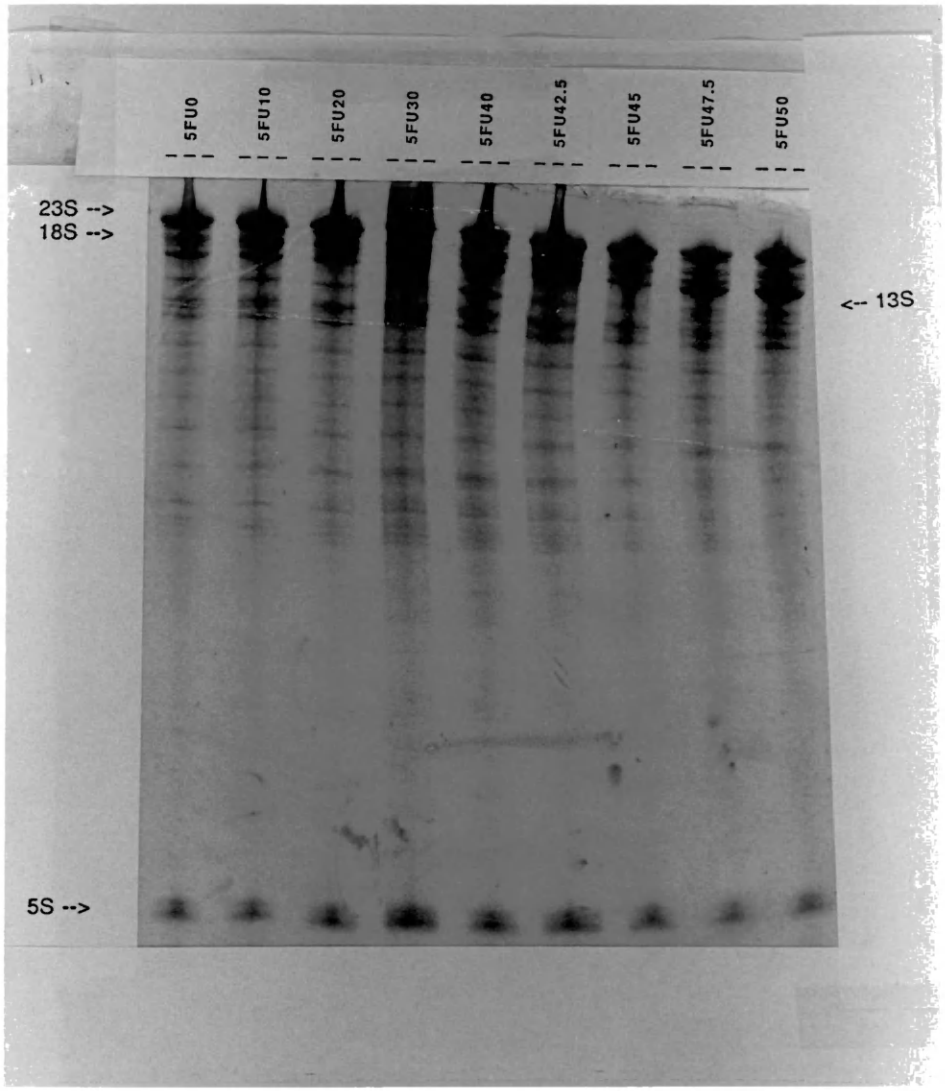

5FU0
5FU10
5FU20
5FU30
5FU40
5FU42.5
5FU45
5FU47.5
5FU50
23S -->
18S -->
<-- 13S
5S -->

Figure 16.

Figure 16. Analysis of rRNA derived from isolated 50S subunits of FUra-treated *E. coli* cells. RNA samples from isolated 50S fraction were purified using the phenol / chloroform extraction procedure described in Method. 10 ug of each sample was loaded onto a 3.75% acrylamide gel stained in methylene blue to detect the RNA bands. Regions showing the breakdown products of the 23S rRNA to 18S, 13S and 5S rRNAs are indicated. (* = Break down of 18S rRNA in 5FU47.5 and 5FU50)

a composite gel. Surprisingly, the samples showed no evidence of any conformational differences (evidenced by the same pattern of migration) (Figure 17). The gel does show that there are differences in the ribosomal component in each of these samples. There is a polyribosome band present in the control sample but not in any of the FUra-treated samples. The 70S fractions from each sample had the same pattern of migration except that the 70S band of the 5FU50 sample is a little darker than the other samples. The 50S band of all FUra-treated samples are not as distinctly separated from the 70S band as in the control sample. The gel shows two types of 30S fractions. One band shows the 30S fraction with the S1 protein and another band without the S1 protein. The 30S fraction in the control sample shows a band corresponding to the 30S fraction with the S1 protein. It did not give a definite band of the S1 minus 30S fraction. In the other samples, this 30S band was somewhat diffuse with increasing FUra concentration. The FUra-treated cells all had a faster migrating 30S band which was the 30S fraction without the S1 protein. This band increased in intensity as FUra concentrations increased.

DOES FUra AFFECT RIBOSOMAL PROTEINS ?

The purpose of this part of the thesis project was to analyze the proteins derived from the ribosomal particles from FUra-treated cells. This analysis was done on a 15% SDS-PAGE gel. Using the ribosomal fractions of all 9 FUra-treated samples, protein samples were prepared by boiling in equal amounts of a 2X solubilization buffer for 5-10 min to remove the rRNA. The protein analysis of the whole ribosomal preparation showed some protein bands that decreased

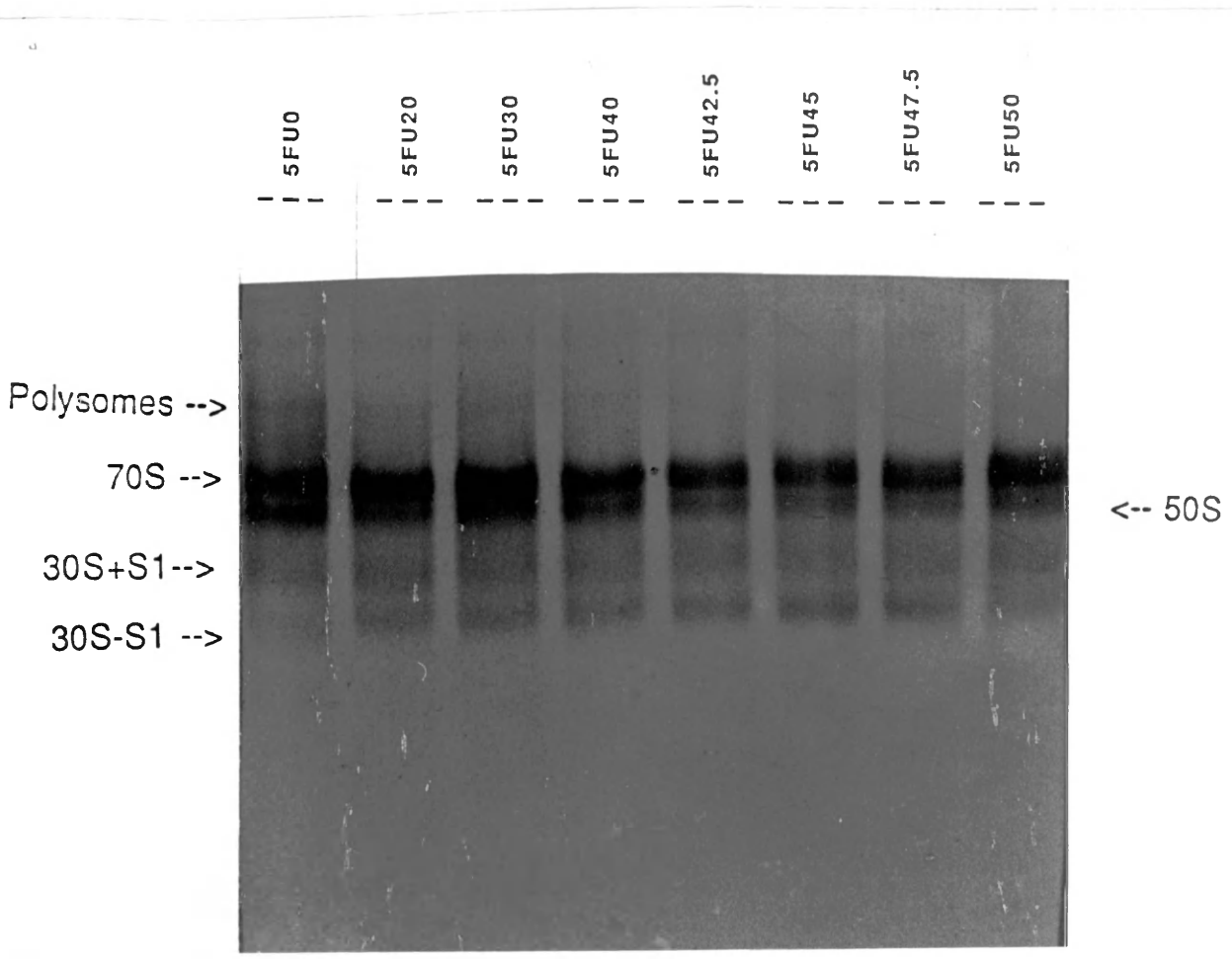


Figure 17. Incorporation of FUra into ribosomal particles in *E. coli* cells. Lysate was fractionated by a 2.25% polyacrylamide / 0.5% agarose composite gel electrophoresis. The ribosomal lysate were prepared as described in Methods as directed by Tapprich & Dahlberg (1990).

in intensity as FUra concentrations increased (Figure 18). Essentially, all ribosomal proteins were present in each sample, but the top set of protein bands, have decreased intensity with increasing FUra. Two protein bands (~57.5 Kd and 56 Kd) are visible only in two samples (5FU0 and 5FU10). Similarly, a protein band below the 45 kd marker band is eliminated at 5FU20. This indicates that with increasing FUra, these proteins are affected.

The protein samples from the 30S fraction gave similar banding patterns (Figure 19). On this gel, the 5FU0, 5FU10, 5FU20 samples have 5 visible protein bands which are not visible in samples with higher FUra concentrations. One of these bands is the S1 protein (65 Kd). This protein band is not visible in samples with higher FUra concentrations. This was consistent with the results of the composite gel (Figure 17).

The same banding pattern was observed on a gel with 50S ribosomal proteins (Figure 20). In this case, there is a protein band absent from all samples except the samples with FUra concentrations of 47.5 and 50 ug/ml (indicated by the asterix). The other ribosomal protein bands were somewhat diffuse under these gel conditions.

IS FUra INCORPORATED INTO rRNA ?

The experiments have shown that FUra affects the growth rate of *E. coli* cells and increases the doubling time of FUra-treated cells when compared to control cell growth rate. They also showed that FUra affects the structure and function of the ribosome. In order to effectively show that FUra was causing these changes in the ribosome, it was necessary to prove that the drug was

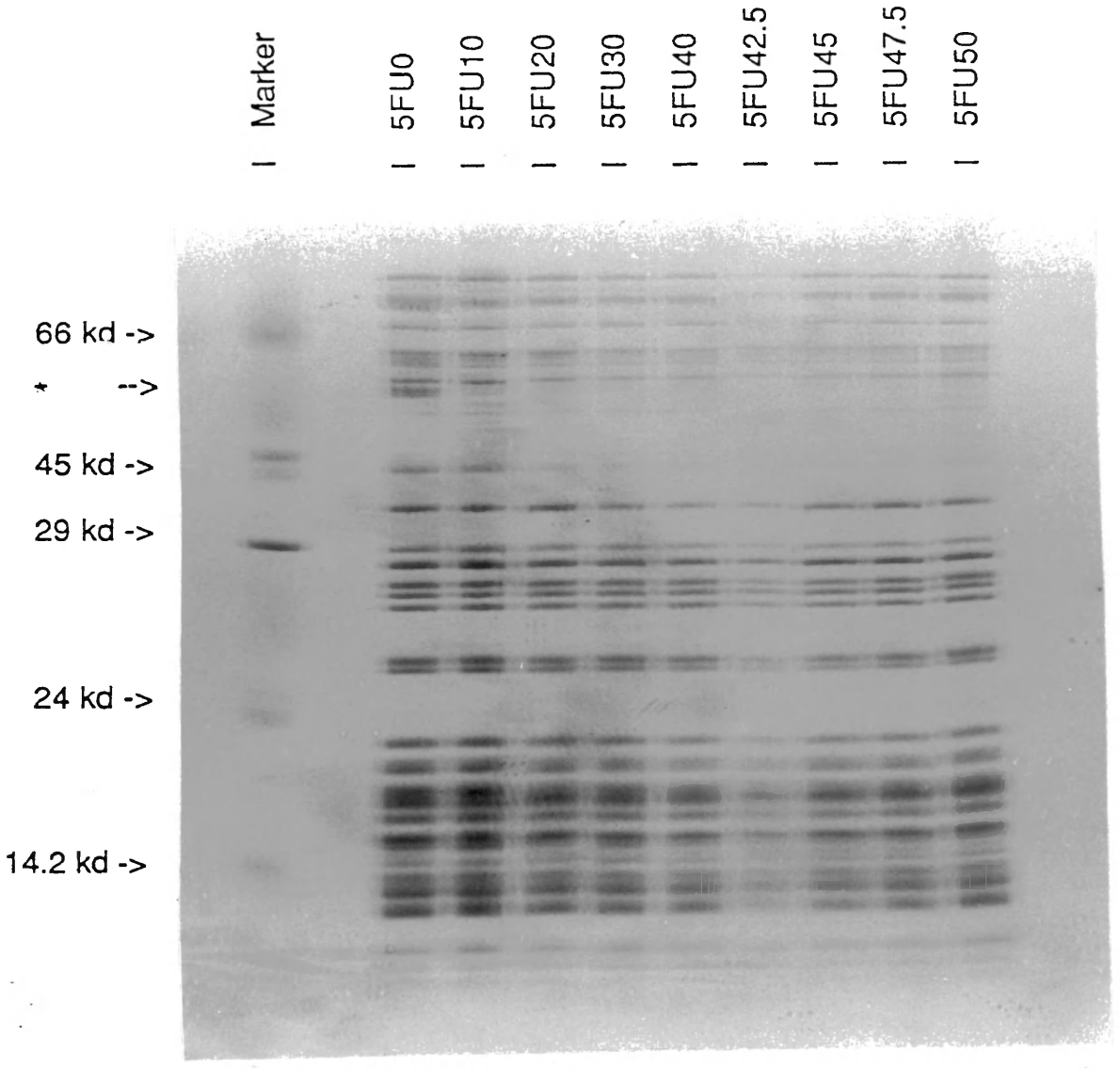


Figure 18. SDS-PAGE one-dimensional analysis of whole ribosomes. Samples were taken directly from ribosomal lysates containing all ribosomal proteins before centrifugation on the 5-30% sucrose gradient as described in Methods. Samples loaded were grown with FUra concentrations between 0 ug/ml (5FU0) and 50 ug/ml (5FU50). (* = protein bands absent in higher FUra concentrations).

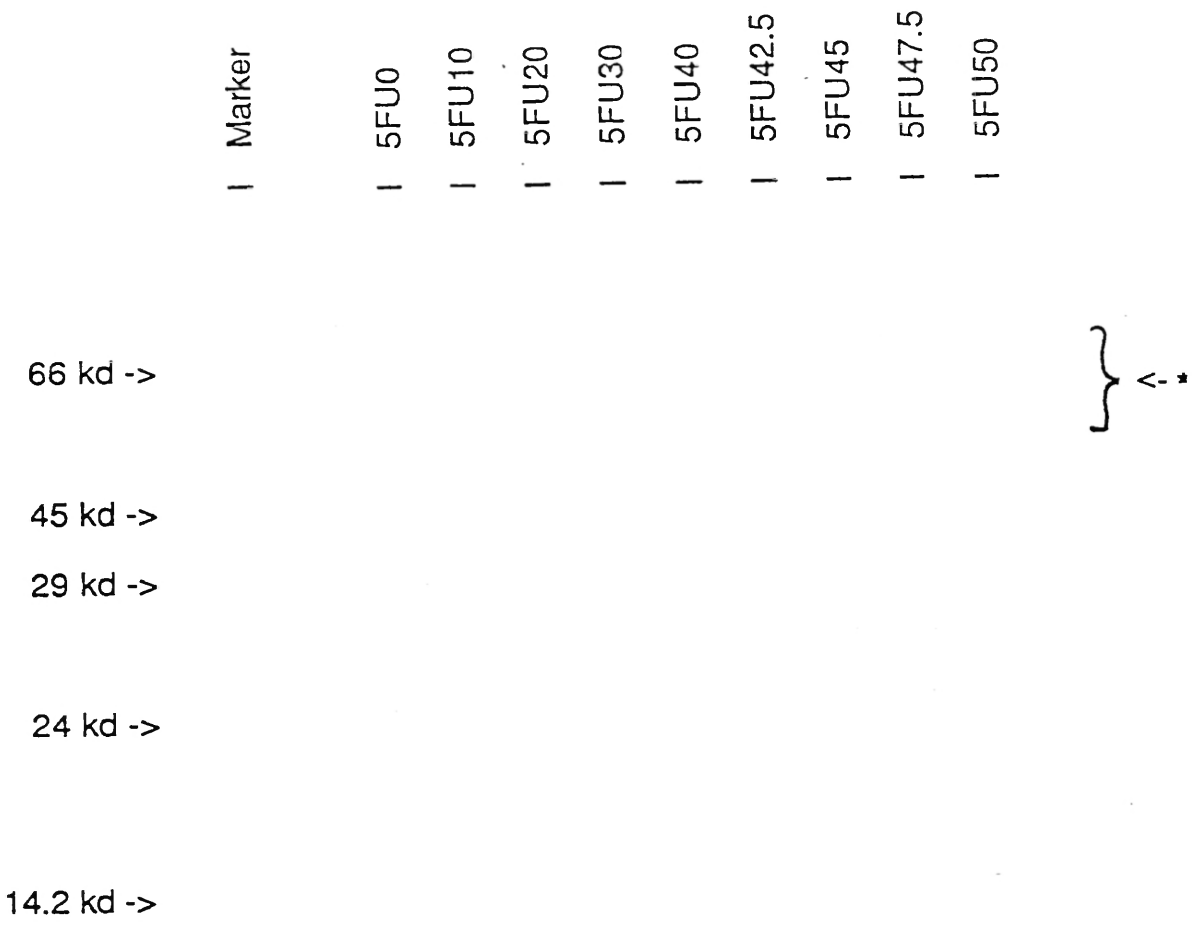


Figure 19. SDS-PAGE one -dimensional analysis of 30S proteins from isolated ribosomal fractions. The 30S ribosomal fraction was centrifuged for 18 hr at 40,000 rpm at 4^{o}C in a Beckman Ti-70 rotor and the pellet was resuspended in TC70S buffer. 30 mg of this sample was prepared as described in Methods and separated by size on a 15% (w/v) polyacrylamide gel and visualized by staining in coomassie blue. Samples were isolated from cells grown in FUra concentrations ranging from 0 ug/ml (5FU0) and 50 ug/ml (5FU50) (* = protein bands absent in higher FUra concentrations).

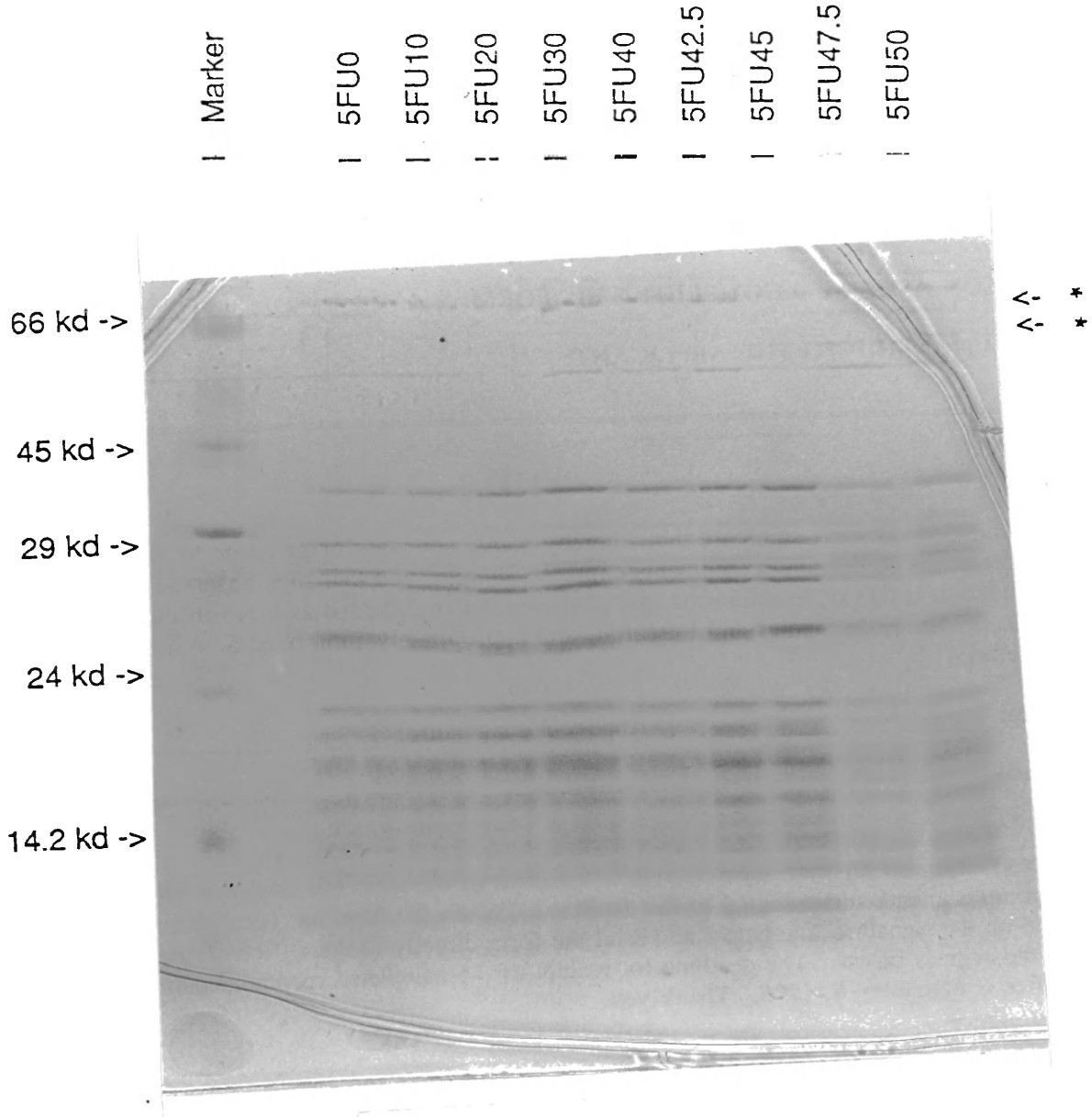


Figure 20. SDS-PAGE one-dimensional analysis of 50S proteins from isolated ribosomal fractions. 50S ribosomal fractions were centrifuged for 18 hr at 40,000 rpm at 4^{o}C in a Beckman Ti-70 rotor and the pellet was resuspended in TC70S buffer. 30 mg of this sample was prepared as described in Methods and separated by size on a 15% (w/v) polyacrylamide gel and was visualized in coomassie blue. Samples were isolated from cells grown in FUra concentrations ranging from 0 ug/ml (5FU0) and 50 ug/ml (5FU50) (* = protein bands present only in higher FUra concentrations).

becoming incorporated into rRNA.

Bacterial cells exposed to 50 ug/ml of ^{3}H-FUra in a time dependent manner were analyzed by allowing the cells to grow for 100 min before a radiolabeled aliquot of the drug was added (6 uci / 50 ug/ml FUra culture medium). The counts produced by the ribosomal fractions indicated that the amount of the drug incorporated into the ribosome in each sample was essentially the same (Table 1).

The absorbancy profiles generated for these samples are similar to those in Figure 14 except that with increased exposure to the drug, the 70S peak got larger and so did the 30S and 50S peaks. This reflects the increase in cell number over time. The first sample shown in Figure 22 (5FU50A) produced almost no polyribosomes and the 70S peak was smaller than those in 5FU50C and 5FU50D (Figures 24 and 25). 5FU50B was somewhat similar to 5FU50A. In 5FU50C (Figure 24) and 5FU50D (Figure 25), the 70S peaks and the polyribosome peaks are comparable to those of the control (Figure 21), but each sample had high numbers of free 30S and 50S subunits.

The fractions generated from each sample was counted on the scintillation counter for 4 mins and also plotted against the absorbance profiles. The findings were interesting. After 60 min of FUra incubation (5FU50A), the radioactivity distribution patterns showed that most of the drug was found in the free subunits. Very little labeled incorporation was seen in the 70S peak.

Increasing the incubation time to 90 min, as in 5FU50B (Figure 23), the radioactivity profiles showed that the amount of labeled drug increased dramatically in the 70S fraction. Very little drug was observed in the

polyribosome fraction. In sample 5FU50C (Figure 24), the amount of incorporated drug into the 50S fraction increased by 28 dpm (tube 19). The 70S fraction also showed a marked increased in incorporation of the drug as characterized by the peak at tube 23. Similarly, the peaks at tubes 31 and 33 showed a slight increase in the polyribosome fraction. In 5FU50D (Figure 25), the 50S fraction showed an increased incorporation of the drug by 14 dpm at tube 17. Drug incorporation into the 70S fraction increased by another 172 dpm characterized at tube 21. Incorporation into the 30S peak seemed to be about the same (tube 13). The peak before the 30S peak also had an increased level of drug incorporation when compared to the others.

The results of the incorporation study indicates that FUra gets incorporated into the rRNA molecules of the ribosome. With time, the amount of FUra incorporated into the RNA is essentially the same. To quantify the relationship between FUra and inhibition of ribosome synthesis, the percentage of radioactivity from ^{3}H-FUra in the total extract that had been associated with ribosomes and ribosomal subunits was calculated i.e.,

$$\frac{\text{sum of dpm in ribosomal fractions only}}{\text{sum of dpm in total cell}} \times 100$$

This indicates that Fura gets incorporated into the rRNA, inhibits association of ribosomal subunits to form the 70S complex leading to the existence of more free subunits and fewer 70S ribosomes. Somehow, this problem is overcome after for a short period of incubation (after 90 min) so that more 70S ribosomes and polyribosomes are formed to continue translation in the cell.

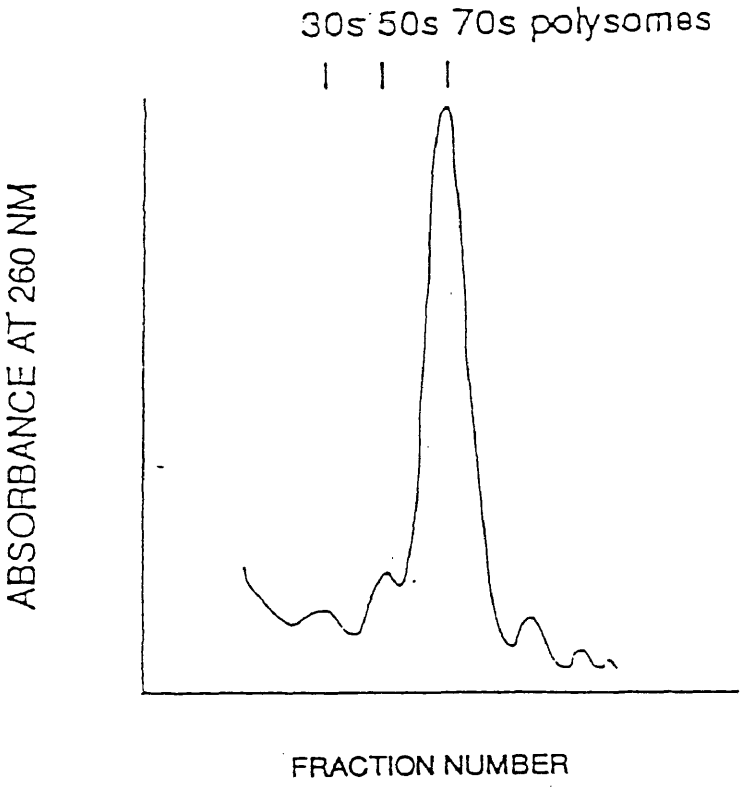
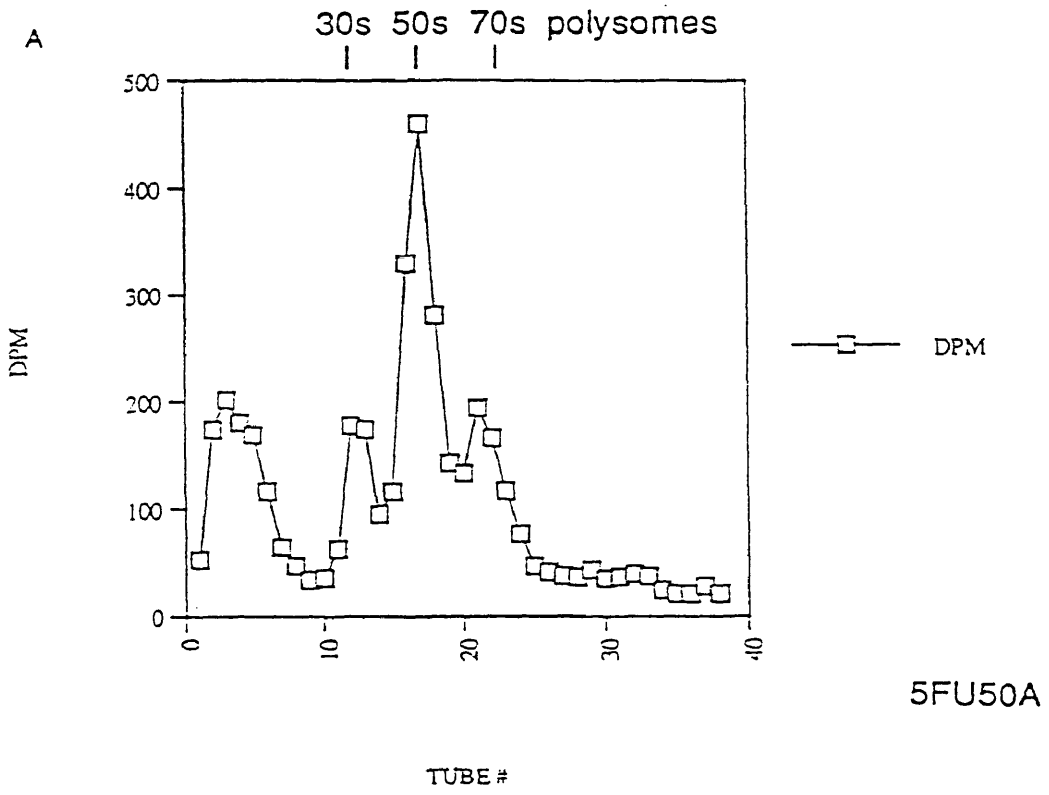


Figure 21. Distribution of absorbance following sucrose gradient analysis of *E. coli* extracts from control cells. Cells were grown in 50 ml LB culture containing no 5FU. Cell lysates were collected as described in Methods according to procedures by Vila *et. al.*, (1994). The pellet was resuspended in 1 ml TKM buffer and loaded onto a 5-30 % (w/v) sucrose gradient under the same buffered conditions. Samples were centrifiuged for 6 hours at 26,000 rpm at $4^{o}C$ in a Beckman SW28 rotor. The polysome fractions were collected as described in Methods.


A
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5FU50A

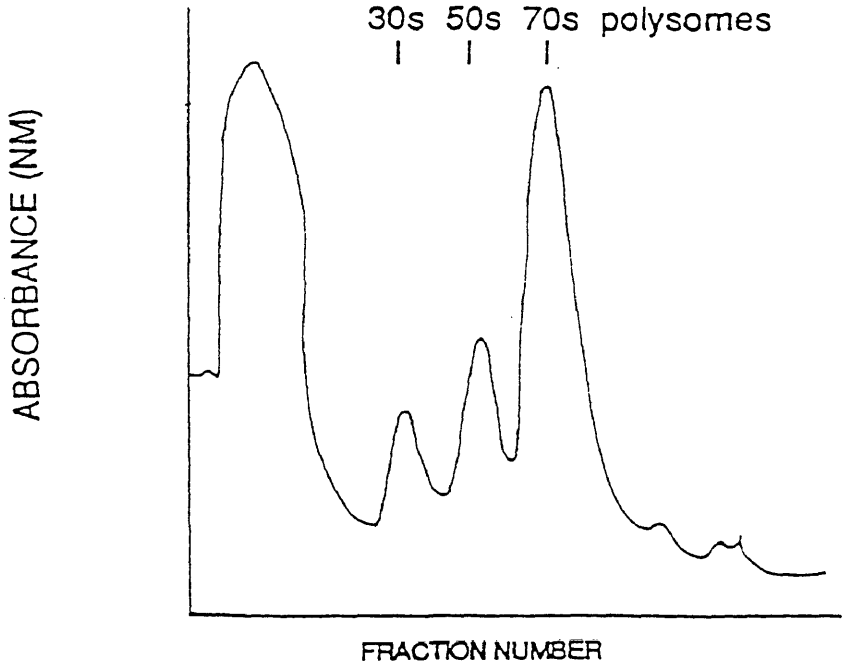
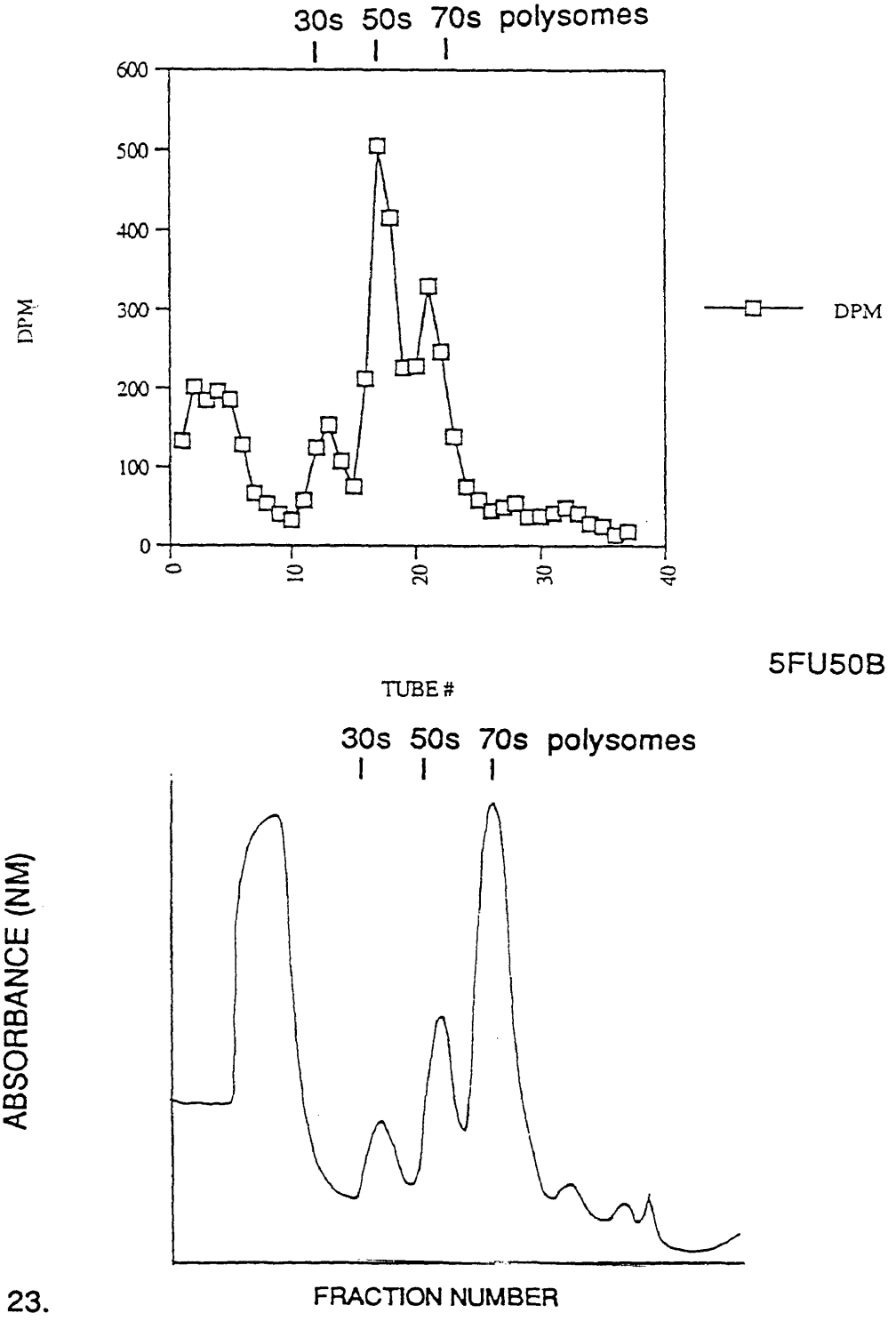

30s 50s 70s polysomes
ABSORBANCE (NM)
FRACTION NUMBER

Figure 22.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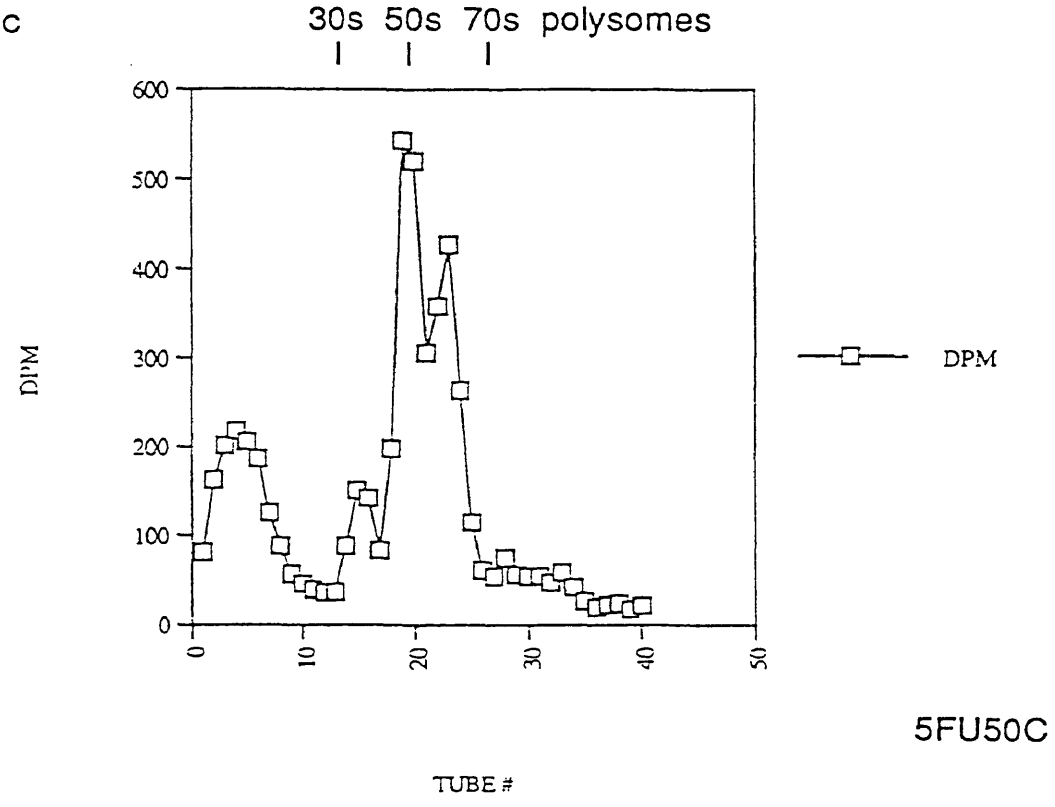
TUBE #
30s 50s 70s polysomes
ABSORBANCE (NM)
FRACTION NUMBER

Figure 23.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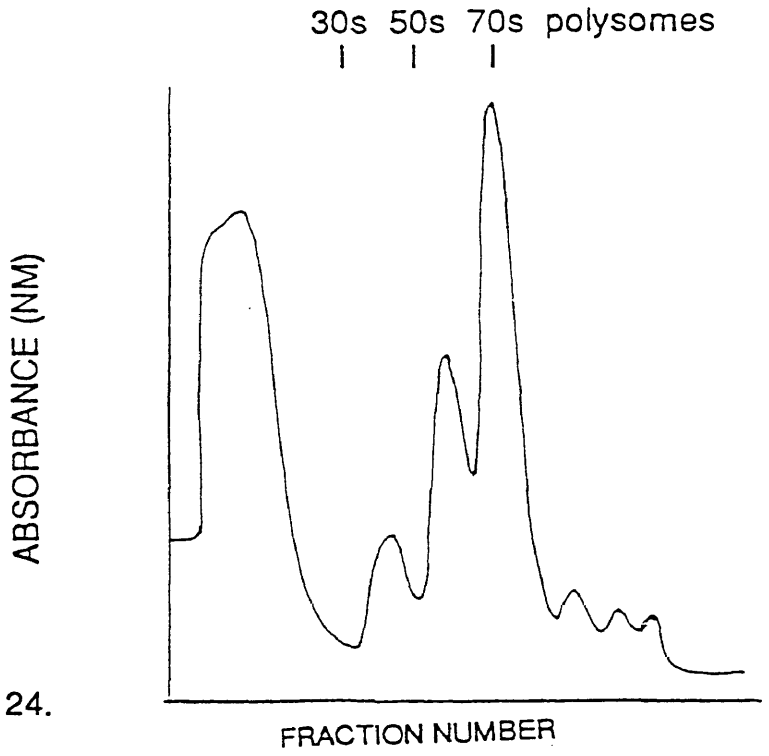
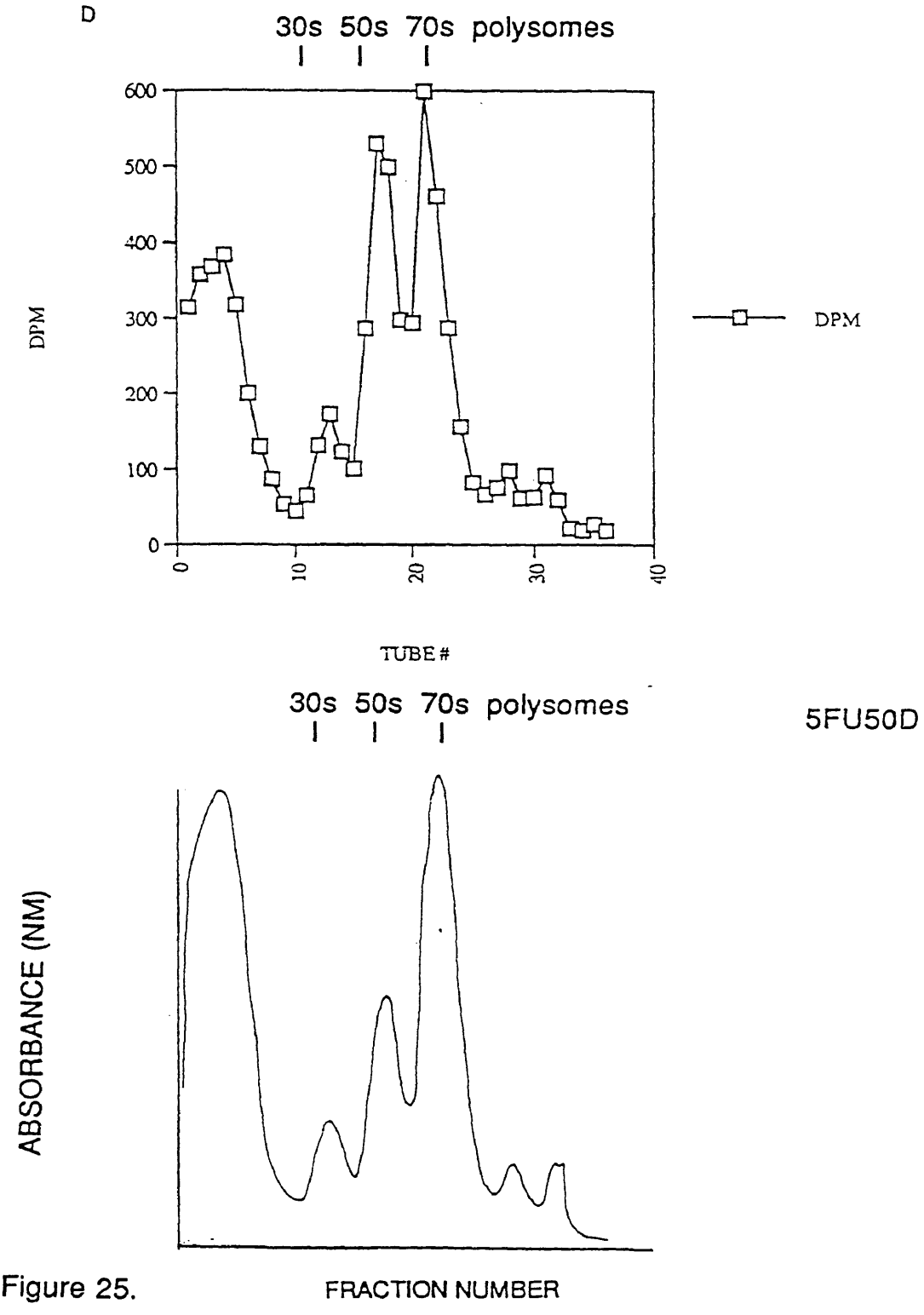

30s 50s 70s polysomes
ABSORBANCE (NM)
FRACTION NUMBER

Figure 24.


D
30s 50s 70s polysomes
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20
30
40
TUBE #
DPM
30s 50s 70s polysomes
5FU50D
ABSORBANCE (NM)
FRACTION NUMBER

Figure 25.

Figures 22-25. Distribution of absorbance and radioactivity following sucrose gradient analysis of *E. coli* extracts from FUra-treated cells. Cells in 50 ml LB cultures were grown up top A_{600} of 0.6 before they were incubated with 6 uci/50 ug/ml FUra for (a) 60 min, (b) 90 min, (c) 120 min and (d) 150 min. Absorbancy at 260 nm, _____; DPM, —□— . Cell lysates were collected as described in Methods according to procedures by Vila *et. al.*, (1994). The pellet was resuspended in 1 ml TKM buffer and loaded onto a 5-30 % (w/v) sucrose gradient under the same buffered conditions. Samples were centrifiuged for 6 hours at 26,000 rpm at 4^{o}C in a Beckman SW28 rotor. The polyribosome fractions were collected and counted as described in Methods.

TABLE

Table 1. Effect of ^{3}H-FUra distribution in ribosomal fraction of *E.coli* cells.

Sample	Incubation Time (min)	Total Cell (dpm)	Ribosomal fraction (dpm)	Total Extract incorporation
Control	0	0.02	0.02	0 %
5FU50A	60	2.91	6.48	74 %
5FU50B	90	2.91	6.39	74 %
5FU50C	120	2.68	7.19	73 %
5FU50D	150	3.41	9.49	68 %

dpm = 10^{+3}

DISCUSSION

FUra is a chemotherapeutic agent that has been shown to have various mechanisms of action within the cell. Several studies have been done to investigate the details of FUra action, but there are still many unanswered questions. For example, studies have shown that FUra affects various RNA species. The focus of this study was to demonstrate the effects of FUra on cellular translation and most importantly, its effects on the structure and function of the ribosome. Most studies on the effects of FUra on the ribosome have approached the issue using short term exposure (one generation time) to the drug (Hahn and Mandel, 1971). This study has expanded on previous studies and has focused on the effects of various FUra concentrations which inhibit cell growth over a long period of time (more than one generation) so as to achieve and maintain a steady level of FUra substitution. This study showed that cells treated with high concentrations of FUra were significantly affected, translation was inhibited and the structure and function of the ribosomes was altered.

The initial experiment done to show the effects of FUra on the growth rate of *E. coli* cells basically supports previous investigations. High concentrations of FUra such as 50 ug/ml or 200 ug/ml have a detrimental effect on the ability of the cells to make functional ribosomes. The fact that there were no obvious differences observed in the growth rates of cells grown in FUra concentrations between 25 ug/ml and 200 ug/ml (Figures 12 and 13) leads to the conclusion that a threshold value exists at which the drug exerts its most powerful effect on the cell. Cells containing 1ug/ml of FUra showed some significant growth

differences from that of the wild-type sample. All the cells containing FUra showed hindered growth rates. Given the results of the ribosome analysis, the cause of altered growth can be interpreted as a diminished level of translation due to the reduced subunit association in these cells.

The sucrose gradient profiles generated from isolated ribosomes indicated that FUra caused an interesting structural defect in ribosomal assembly. The absorbance profile of cells containing 10 ug/ml FUra concentration did not show significant differences from that generated by the control sample. However, cells with FUra concentrations ranging from 20 - 40 ug/ml gave profiles that showed an increasing amount of free subunits and fewer numbers of 70S complexes. The ribosomes from cells grown in 5FU40 to 5FU50 (Figure 14) all showed a tremendous decrease in subunit association (i.e. more free 30S and 50S subunits). Somewhat similar results were obtained in previous experiments done by Hahn and Mandel (1971). However, in their experiment, they used 48 uM of FUra was utilized, and allowed the cells to reach an absorbance value (A_{540} nm) of 0.1. In the present experiment, the cells were allowed to grow until an absorbance value ($A_{600\ nm}$) of 0.6 was reached before they were harvested. The difference in the current results when compared to previous experiments is that at high FUra concentrations, the absorbance profiles indicate structurally altered ribosomes.

The extreme broadening of the peaks in the profiles generated by the sucrose gradients showed the changing conformation of the ribosome as a result of the drug. The ability of the subunits to associate properly is greatly decreased with increasing drug concentration. Growth data (Figure 12) showed

that ribosomes from these cells were seriously hindered from performing normally. Given the sucrose gradient profiles, this can be interpreted as a decreased translational competence of the altered ribosomes. Despite the clear and signifiant alteration in ribosome conformation, cells were still capable of growing to A_{600} of 0.6 when given enough time. Although the growth rate experiment (Figure 12) seems to indicate no growth, there was some growth observed. Cells without FUra were harvested after just 2.5 hours. Typically, it took the other FUra treated cultures (5FU30 - 5FU50) between 4 and 5 hours to reach an absorbance value ($A_{600\ nm}$) of 0.6 before they were harvested. Thus, the overall profiles showed that with increasing FUra concentrations, translational rates of these ribosomes was greatly reduced leading to slower growth rates than those observed in the wild-type cells.

To compliment the experiment on isolated ribosomes, the translational apparatus from whole cell lysates was also examined. As shown in the polyribosome profiles in Figure 15, even at high concentrations of FUra, there are translating ribosomes and polyribosomes. This is somewhat in contrast to the previous results in Figure 14 which showed that at concentrations between 40 and 50 ug/ml, naked ribosomes gave characteristically different profiles from the wild-type. Basically, the isolated ribosomes were not at all characteristic of functional ribosomes at such high concentrations. In the case of the cell lysate, the translating pool of FUra-treated cells was analysed and the profiles gave absorbance profiles with distinct 30S, 50S and 70S peaks (Figure 15).

Comparing the two procedures (ribosome preparation and polysome preparation), it is obvious that there are translating ribosomes in cells treated

with high FUra concentrations as seen in Figure 15. This is not very obvious in the absorbance profiles of 5FU47.5 and 5FU50 in Figure 14 which shows a merged peak in these samples. As mentioned in the previous section, in the preparation of ribosomes, 0.5 M NH_4Cl in Buffer B was used to remove cellular factors so as to analyze the ribosomes in these FUra-treated cells. In the preparation of polyribosomes, ribosomes were assayed in the presence of all cellular factors. This serves to illustrate the important effects certain cellular factors exert on the structure and function of the ribosome. Hence, FUra must play an important role in inhibiting some cellular factor that indirectly or directly affects the structure and the function of the ribosome during translation.

The structural and functional effects of FUra on rRNA was also observed. FUra incorporation into the 45S rRNA of eukaryotic cells is known to cause inhibited maturation of precursor rRNA to make large ribosomal subunits (Kanamaru et. al., 1986). In prokaryotic cells, it has been shown that FUra incorporation into 23S rRNA inhibits processing to a much greater extent than in the 16S rRNA of the 30S subunit (Hahn and Mandel, 1971). Figure 16 shows a marked increase in the degradation of 23S rRNA with increasing FUra concentrations. This can be interpreted as either poor assembly or as shorter half-life. These samples were taken from FUra-treated cells in the preparation of ribosomes in TC70S buffer. This study supports previous data showing that the stability of 16S rRNA is not significantly affected by FUra incorporation. However, the darkened bands in Figure 16 indicate that the drug has a definite effect on the 23S rRNA of the large subunit. It is possible that the effects of the drug on the 50S subunit may cause it to become unable to associate with the

30S ribosomal fraction to form the 70S complex. Unfortunately, this was not directly tested. For some reason, FUra affects the 50S ribosomal subunit more than the 30S subunit and the reason for this is still unclear.

Previous studies have shown conflicting data with this particular study of 5-fluorouracil. Armstrong et. al. (1986), showed that cells treated with 10 uM or 100 uM FUra plus thymidine showed no effect on the 18S and 28S rRNA in eukaryotic cells even though both concentrations gave 100% clonal growth inhibition. Parker and Cheng (1990) explained this by the fact that mammalian RNA is stable and so even if no new RNA was made during exposure to FUra, the residual RNA may have been able to continue the cells' protein synthetic needs. Similar results were observed in a study done by
Dolnick and Pink (1983).

In this present study, the results of the incorporation studies gave several insights on the previous experiments, but it also posed many new questions. Two previous experiments that become more clear as a result of the incorporation study are the growth rate experiments in Figure 12 and 13 and the sucrose gradients of polyribosomes in Figure 15. In the growth rate experiment, even though 200 ug/ml FUra was used in that experiment (5FU200B), similar results were produced when 50 ug/ml of FUra is used to assess growth rate in cells (Figure 12) ie., no growth was observed. The incorporation study showed that when ^{3}H-FUra was added to healthy growing cells after 100 min of growth, newly synthesized ribosomes containing FUra did not immediately enter the 70S or polyribosomes. The data in Figure 22 showed that the already formed 70S complexes had very little incorporation after 1 hour of drug incubation.

5FU50A showed that once the drug was added, it became more incorporated into the newly synthesized RNA (mostly the free subunits). These newly synthesized RNAs were continuously recycled and as a result were kept out of the translating pool of functional ribosomal RNA. Polyribosomes and 70S ribosomes in this sample, also had very little drug incorporated.

However, with increased incubation time, more of the drug was found in the 70S fraction. Specifically, after 2.5 hrs of incubation, the bulk of the labeled drug was found in the 70S fraction even though the absorbance peak was of a similar height as that of the control sample. The polyribosome peaks also showed increased incorporation of the drug compared to the earlier samples (5FU50B and 5FU50C). Likewise, the absorbance profiles in 5FU50C and 5FU50D were rich in free subunits. This seems to be a paradox. In Figure 15, increased exposure to the drug shows that the growth rate of cells is continously hindered and the number of 70S ribosomes and polyribosomes was decreased. However, in Figures 22 through 25, the ribosomes seemed to be recovering from the drug effects and forming particles that translate normally. A probable explanation for the latter results is that after the addition of the drug, normal ribosomes in these cells continued to help the cells survive, but growth is at a much slower pace in an overall sense. With time, the number of normally replicating cells increase as well as newly synthesized and defective ribosomes containing the drug which also increase at a much slower pace. Thus, with continued exposure, the drug has to become incorporated in the normal translating pool of ribosomes which explains the increased incorporation of the drug in the 70S fraction and polysomes. In addition, as explained earlier, the

continued exposure of the cells to FUra indicates that some cellular factor may be transiently affected by the drug which is overcome so that the cells are able to allow rRNA maturaton (Parker and Cheng, 1990). All the evidence from this study suggests that this recovery over time is not a ribosomal effect. Perhaps, the S1 protein in the FUra-treated cells is transiently affected so that with continued exposure to the drug, the cell alters the production of S1, thereby compensating for the damaged subunits. In addition, the overall incorporation of the drug into the RNA in each cell sample was about the same even though in 5FU50D, there seemed to be a higher incorporation of the drug in this sample (Table 1). Finally, the differences observed in these two experiments, the polyribosomes prepared in Figure 15 and those prepared in the incorporation study, can also be explained by the fact that in the former preparation, FUra was added to the media before inoculation while in the latter experiment, addition of the drug took place 100 min after inoculation.

The effect of FUra on the ribosome structure was observed in the analysis of FUra-treated, naked ribosomes. The absorbance profiles of 5FU47.5 and 5FU50 indicated that the ribosomes in these samples were conformationally different from those found in other samples with no FUra or low concentrations of the drug (Figure 14). The composite gel experiment was done to study the conformational changes in the ribosomal structure of FUra-treated cells. It gave no such results. While this was somewhat surprising, it is possible that the conformational changes observed on the gradient profiles were not significant enough to be detected by the composite gel. However, the composite gel experiment did give other very important information about these

FUra-treated ribosomal particles. As seen in Figure 17, the separation pattern of ribosomes from the FUra-treated cells were different from the control sample. The absence of polysomes in any of the FUra samples correlates very well with the polyribosome profiles shown in Figure 15. As mentioned earlier, the FUra-treated samples had a different 30S band, i.e the 30S band without the S1 protein. The S1 protein is an important protein for initiation during translation. This means that FUra inhibits the binding of this protein which is necessary to allow the 30S subunit to become bound to the 50S subunit to form the 70S complex during translation.

The observations seen on the protein gels (Figures 18-20) showed few differences in protein composition, however the protein bands had different intensities suggesting that the asssembly of certain ribosomal proteins were indeed affected by FUra. In Figure 18, cellular proteins associated with unfolded particles are present but, with increasing drug concentration, these protein bands decrease in intensity until some of them are no longer visible in the 5FU50 sample. This indicates that with increased drug concentration, the production of these proteins is hindered. Also, the absence of the S1 protein in these samples may also indicate the that the 30S subunit had difficulty during its association with the 50S subunit. Without the S1 protein initiation cannot occur.

The protein analysis of the 50S fraction also gave similar results to the previous experiment (Figure 20). In this experiment, some of the protein associated with ribosomes are not visible on the gel. A band corresponding to a 67 kd protein is present in all samples, but in this case, the intensity of this

protein band is increased with increasing FUra concentrations. On the other hand, two protein bands (62 kd and 57.5 kd) were absent in all samples except in 5FU47.5 and 5FU50. These protein bands are probably not normal ribosomal proteins. An explanation for the increased production of these particles in these FUra-treated cells is unclear. Probably, the drug targets the cell in such a way as to cause an increased production of these ribosomal particles during translation. Basically, all the other ribosomal proteins were present in the ribosome.

This project has shown that there are significant affects of FUra on the ribosome and on translation using *E. coli* cells as a model. It has been shown that the ribosome is severely affected by high concentratrions of FUra. As a result, translation in these cells is hindered. The results of the absorbance profiles showed the structural effects of the drug on the ribosome even though such structural effects were not observed using the composite gel technique. Several other experiments however, did show complimentary results which either supported or expanded on previous studies. Most importantly, the results of this study gave several conclusions. Firstly, at high concentrations of FUra, the translational apparatus of the cell is severely hindered. Secondly, certain important proteins such as the S1 protein which is involved in the translational process is affected by the drug. Thirdly, some cellular proteins associated with the ribosome were affected by FUra.

The incorporation study also gave a better indication of what actually occurs in a real setting where the drug is administered to already growing tumor cells. An aspect of the drug that was not addresed in this study is the

cytotoxicity of FUra. However, the fact that FUra becomes incorporated into normal translating ribosomes with continued exposure leads one to understand how the drug must be monitored when administered to patients. A drawback of the drug is keeping it localized so as not to affect other normal functioning cells in close proximity to the tumor cells that may or will be affected by the drug. The incorporation assay did show that FUra was effectively incorporated in these cells and it helped to understand what was actually happening in these cells.

More specific studies will have to be done to better target the action of FUra in the translational process of cells and to continue the pursuit of more knowledge and answers about 5-fluorouracil. This research project has brought about some critical answers to the study of FUra. It will be interesting to observe other research findings in this area.

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