Effect of 5-Fluorouracil on RNA Metabolism in Novikoff Hepatoma Cells

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ABSTRACT

The resistance of a Novikoff hepatoma cell line (N1-S1/FdUrd) to 5-fluorouracil (FUra) was reversed by the inclusion of inosine in the culture medium. As the concentration of inosine in the medium was increased, there was a marked increase in the uptake of [14C]FUra and conversion to nucleotides with a corresponding increase in the incorporation into RNA. While FUra alone had no effect on this resistant cell line, the combination of FUra plus inosine altered the levels of ribose 1-phosphate but not 5-phosphoribosyl 1-pyrophosphate, altered the maturation of precursor ribosomal RNA by blocking the formation of 18S RNA, altered the methylation of the ribosomal RNA, and caused inhibition of the growth of these cells. No evidence was obtained that fluorodeoxyuridine 5'-monophosphate was formed in the N1-S1/FdUrd cells as a result of treatment with FUra plus inosine. In addition, the metabolism of [3H]deoxycytidine in the presence of FUra plus inosine in the intact N1-S1/FdUrd cells did not indicate significant inhibition of thymidylate synthetase as evidenced by the levels of deoxyuridine 5'-monophosphate or conversion to thymidine 5'-monophosphate.

INTRODUCTION

FUra3 is currently in use in the treatment of certain types of human tumors (6). For FUra to be effective as a cytotoxic agent, it must be metabolized by the cells to the nucleotide level. There are several pathways by which FUra can be activated to the nucleotide level. These pathways are shown in Chart 1. Through the reaction catalyzed by pyrimidine nucleoside phosphorylase, FUra and ribose-1-P react to yield FUr, which can then be phosphorylated to 5-fluorouridine 5'-monophosphate, FUDP, and FUTP. Alternatively, FUra can react with PRPP via pyrimidine phosphoribosyltransferase to yield 5-fluorouridine 5'-monophosphate, which in turn is phosphorylated to FUDP and FUTP. FdUMP can be formed either by the phosphorylase and kinase pathway utilizing deoxyribose 1-phosphate or by the reduction of FUDP via ribonucleotide reductase. The conversion of FUra to FUTP leads to the incorporation of FUra into RNA, while the conversion to FdUMP yields a potent inhibitor of thymidylate synthetase. The inhibition of thymidylate synthetase by FdUMP has long been proposed and accepted as the site of action of FUra (6). However, recent studies have indicated that the incorporation of FUra into RNA has important metabolic consequences for the cell, leading to cytotoxicity (4, 9, 10, 13, 18, 19, 21). Wilkinson and Pitot (19) showed that the incorporation of FUra into RNA caused the inhibition of rRNA maturation. Using Novikoff hepatoma cell lines which were sensitive and resistant to FUra, Wilkinson and Crumley (18) showed that the resistant cell line, which had been originally derived as a line resistant to FdUrd because of a thymidine kinase deficiency (11), also lacked the ability to convert FUra to FUra ribonucleotides. Gotto et al. (5) showed that inosine stimulated uracil and FUra incorporation into nucleotides and RNA of Ehrlich tumor cells. Using this approach, it was possible to markedly stimulate the uptake of FUra into the acid-soluble fraction as FUra nucleotides (primarily as FUTP) and into the RNA of the FUra-resistant Novikoff hepatoma cells (4). The presence of inosine in the culture medium reversed the FUra resistance in this Novikoff hepatoma cell line.

In the present study, experiments were carried out using the N1-S1/FdUrd cell line to determine, with the use of inosine, the relative importance of the incorporation of FUra into RNA and the inhibition of the thymidylate synthetase step as the major sites of action of FUra metabolites.

MATERIALS AND METHODS

Tissue Culture. The Novikoff hepatoma cell lines N1-S1 and N1-S1/FdUrd were obtained from Dr. Van R. Potter, McArdle Laboratory for Cancer Research, Madison, Wis., and were derived originally from a chemically induced solid tumor (15).

The cell lines were grown in Medium S-69 supplemented with calf serum and Pluronic F-68, as previously described (19). Cell density measurements were made using a Model 6300A Bio/Physics Cytograph.

Incorporation of Labeled Precursors into Cells. N1-S1 or N1-S1/FdUrd cells (0.7 to 1.2 x 10^6/ml) in log phase were collected by centrifugation and resuspended in one-fifth the original volume in fresh culture medium. The labeled precursors used were: [14C]FUra (0.5 μCi/ml; 50 mCi/mmol), [14C]FdUrd (0.5 μCi/ml; 52 mCi/mmol), and [U-14C]guanosine (0.2 μCi/ml; 508 mCi/mmol). The cells were incubated for 1.5 hr at 37° in the presence of the labeled precursor and in the presence and absence of inosine. The cells were collected by centrifugation, and the cell pellet was treated as described for the specific experiments.

Isolation of Acid-soluble RNA and DNA Fractions from Labeled Cells. The cells, after incubation with labeled precursor, were collected by centrifugation and subjected to the Schmidt-Thannhauser procedure (17) using 6% perchloric acid. The acid-soluble fraction was neutralized with KOH and the supernatant fluid was lyophilized. RNA and DNA concentrations were determined by the methods of Hurlbert et al. (7) and Burton (3), respectively. All cell incubations were set up in triplicate.

For the determination of the percentage of conversion of...
[\[^{14}C\]Fura to \[^{14}C\]Fura nucleotides, duplicate aliquots of the neutralized acid-soluble fractions were spotted on separate DEAE-cellulose discs. One of the discs was washed with 1 mM ammonium formate to remove the \[^{14}C\]Fura. The washed discs represent the \[^{14}C\]Fura nucleotides, while the unwashed discs represent the total acid-soluble fraction. From the counts on the washed and unwashed discs, the percentage of conversion to nucleotide was calculated. This was done for each concentration of inosine used.

**Isolation and Analysis of Intact RNA.** RNA was isolated from the labeled cells by the hot phenol method of Wilkinson and Pitot (19). Sucrose gradient analysis of the RNA was carried out on linear sucrose gradients (5 to 47%). RNA (5 A\textsubscript{260} units) was layered onto the gradients and run for 18 hr at 26,000 rpm in an SW27 rotor at 4\(^\circ\). The gradients were fractionated using an Isco gradient fractionator. Fractions (0.6 ml) were collected, after passing through the UV monitor, directly into scintillation vials. Water (1.4 ml) was added to each vial followed by 10 ml of scintillation fluid (Type 3a70B; Research Products International).

**Determination of Nucleoside Triphosphate, Ribose-1-P, and PRPP in N1-S1 and N1-S1/FdUrd Cells.** Cell pellets (approximately 90 \times 10\(^6\) cells) of the N1-S1 and N1-S1/FdUrd cell lines were extracted with 60% methanol. The methanol extracts were lyophilized and dissolved in 1 ml H\textsubscript{2}O.

Aliquots (40 \mu l) were analyzed by HPLC using a Partisil SAX column (Whatman, Inc., Clifton, N. J.; 25 x 0.46 cm) with 0.5 M NH\textsubscript{4}H\textsubscript{2}PO\textsubscript{4}, pH 4.82, as the eluting buffer. Under these conditions, all components other than the triphosphates elute in the void volume. Each sample was analyzed in triplicate. The flow rate was 2 ml/min. A Lab Data Control HPLC equipped with a Spectra Physics Minigrator was used.

Ribose-1-P levels were determined enzymatically. The reaction mixture consisted of 0.19 mM \[^{14}C\]hypoxanthine (1.0 \mu Cl/tube), 0.06 mM sodium citrate buffer (pH 6.3), and nucleoside phosphorylase. The reactions were started with the addition of aliquots of known concentrations of ribose-1-P or the methanol extracts. The reactions were carried out for 1 hr at 37\(^\circ\). After the reactions were stopped by heating in a boiling H\textsubscript{2}O bath, aliquots (20 \mu l) were spotted on cellulose plates and the chromatogram was developed with H\textsubscript{2}O to separate the hypoxanthine from inosine.

The hypoxanthine and inosine areas were detected under UV light, cut out, and counted for radioactivity. Controls containing known amounts of ribose-1-P were run simultaneously to prepare a standard curve.

PRPP levels were also determined enzymatically. The reaction mixture contained 50 mM Tris-HCl (pH 7.8), 50 mM KCl, 6 mM MgCl\textsubscript{2}, 0.1 mM EDTA, 2 mM dithioerythritol, 0.06 mM \[^{14}C\]adenine (0.1 \mu Cl/tube), and adenine phosphoribosyltransferase prepared from Ehrlich tumor cells. The reactions were started by the addition of known amounts of PRPP or the methanol extract. The reactions were carried out at 37\(^\circ\) for 0.5 hr and stopped by boiling. Aliquots (50 \mu l) were spotted on Whatman No. 3 MM chromatography paper and developed overnight with butanol:H\textsubscript{2}O (86:14). AMP remained at the origin, which was cut out and measured for radioactivity.

**Incorporation of \[^{methyl-14}C\]Methionine into RNA.** The N1-S1 (1.24 \times 10\(^6\)/ml) and N1-S1/FdUrd (1.02 \times 10\(^6\)/ml) cells were collected by centrifugation and washed twice with methionine-free medium. The cell pellets were resuspended in one-fifth the original volume of methionine-free medium. Aliquots of cells (5 ml) were put into flasks in the presence and absence of inosine (1 mM) and Fura (0.01 mM). The cells were incubated for 1.5 hr at 37\(^\circ\). \[^{methyl-14}C\]Methionine (0.2 \mu Ci/ml; 54.06 mCi/mmol) was added to each flask, and the incubations were carried out for an additional 1.5 hr.

**Attempts to Determine FdUMP.** The N1-S1/FdUrd cells (0.72 \times 10\(^6\)/ml) were collected by centrifugation and resuspended in one-fifth the original volume. Aliquots of cells (5 ml) were put into culture flasks containing \[^{14}C\]Fura (0.5 \mu Ci/ml) and various concentrations of inosine (0 to 5 mM). The cells were incubated for 2 hr at 37\(^\circ\). The acid-soluble fraction was prepared using 1 mM acetic acid (14). The acetic acid extract was lyophilized, and the dried samples were dissolved in 200 \mu l of a mixture containing 0.06 mM Tris (pH 9.0), 0.02 mM MgCl\textsubscript{2}, and crude snake venom (Crota1us atrox; 1 mg). The samples were incubated for 4 hr at 37\(^\circ\), and the reactions were stopped by heating in a boiling H\textsubscript{2}O bath. The samples were centrifuged, and aliquots (50 \mu l) of the supernatant fluids were used for analysis by HPLC.

HPLC analysis was carried out using a C\textsubscript{18,5}Bondapak column (3.9 x 300 mm) from Waters' Associates, Inc., Milford, Mass. H\textsubscript{2}O was the eluting solvent and was pumped at a flow rate of 2 ml/min. Fura, FUrA, and FdUrd standards eluted with retention times of 3.0, 6.7, and 9.9 min, respectively (2). After passage through the UV monitor, the effluents were collected directly into scintillation vials (1-ml fractions).

In the second method, the enzymatic assay of Myers et al. (12) was used. The nucleotide pool was extracted from the N1-S1/FdUrd cells with 1 mM acetic acid (14). Thymidylate synthetase from Lactobacillus casei was used. Standard curves utilizing varying concentrations of dUMP were prepared for determination of the dUMP levels. The reactions were started by the addition of the neutralized acid-soluble extract. For determination of the FdUMP levels in these extracts, after a reaction period of 10 min, saturating levels of dUMP were added, and the degree of inhibition of thymidylate synthetase was determined. From these studies, both the dUMP and FdUMP levels could be determined in each sample.

**Metabolism of \[^{5-3H}\]Deoxycytidine in Intact N1-S1 and S1/FdUrd Cell Lines.** The sensitive (N1-S1) and Fura-resistant (N1-S1/FdUrd) cell lines were incubated with \[^{5-3H}\]deoxycytidine in the presence and absence of Fura and inosine. The acid-soluble fractions were prepared using 6% perchloric acid. They were heated for 30 min in a boiling H\textsubscript{2}O bath to hydrolyze the pyrimidine nucleoside di- and triphosphates to the monophosphate level. The samples were cooled and neutralized with KOH. Aliquots (1 ml) were put over Dowex 50-H\textsuperscript{+} columns (6 x 70 mm) to separate dUMP and \(^{3}\)H\textsubscript{2}O (arising from conversion of dUMP to dTMP) from dCMP. The columns were washed with 4 ml of H\textsubscript{2}O. Aliquots of the effluent from the Dowex 50-H\textsuperscript{+} columns were then treated with charcoal to
remove dUMP from the $^3$H$_2$O. An aliquot of the supernatant fluid was used to determine the amount of $^3$H$_2$O which was formed as the result of dTMP synthesis.

**Growth Curves.** Flasks containing fresh medium (25 ml) and the various agents (±inosine, 0.5 mM; ±FUra, 0.01 mM; or ±uridine, 0.1 mM) were inoculated with $1 \times 10^6$ cells in log phase. The cells were incubated at 37°C. Aliquots (1 ml) were centrifuged, and the cell pellet was suspended in 2 ml of citric acid (8.3%) for counting.

**Materials.** The radiochemicals used in these experiments were purchased from the following suppliers: $[^{14}C]$FUra (50 mCi/mmoll, $[^{14}C]$FdUrd (52 mCi/mmoll), $[^{14}C]$FdUMP (50 mCi/mmoll) from Moravek Biochemicals, City of Industry, Calif.; $[^{14}C]$guanosine (508 mCi/mmoll) and [methyl-$^{14}$C]methionine (54.06 mCi/mmoll) from New England Nuclear, Boston, Mass.; [5-$^{3}$H]deoxycytidine (20 Ci/mmoll) from Amersham/Searle Corp., Arlington Heights, Ill.

Swim's Medium 77 and the calf serum were purchased from Grand Island Biological Co., Grand Island, N. Y. The Pluronic F-68 was a gift from Wyandotte Chemical Co., Wyandotte, Mich. The biochemicals used in these studies were purchased from Sigma Chemical Co., St. Louis, Mo. Nucleoside phosphorylase was purchased from Sigma. Thymidyate synthetase (L. casei) was purchased from the New England Enzyme Center, Tufts University School of Medicine, Boston, Mass.

**RESULTS**

**Ribonucleotide Pools in Sensitive and Resistant Novikoff Hepatoma Cells.** The N1-S1/FdUrd cells, which have been characterized as thymidine kinase-deficient cells (11), also have other metabolic alterations (4, 18). Among these is an alteration in the concentrations of the ribonucleotides phosphates in the cells. As seen in Table 1, there is a marked increase in the concentration of the UTP level relative to CTP in the resistant cells compared to the sensitive cells. This increased level of UTP could play a role in the resistance to FUra, in that the FUTP generated in the cell would be competing with higher levels of UTP for the various metabolic pathways.

**Riboase-1-P and PRPP Levels in FUra-sensitive and -resistant Cells.** In order to determine the effect of inosine on the pathways by which FUra could be activated to the nucleotide level, ribose-1-P and PRPP levels were determined in both cell lines and under various incubation conditions. These data are given in Table 2. The presence of 0.5 mM inosine in the culture medium caused a 5- to 7-fold increase in the ribose-1-P levels in both cell lines, while the PRPP levels did not change. FUra had no effect on the levels of ribose-1-P or PRPP. In the presence of both FUra and inosine, the increase in ribose-1-P was again observed with no change in the level of PRPP.

**Effect of Inosine on [14C]FUra Uptake, Conversion to Nucleotides, and Incorporation into RNA.** We had earlier shown that inosine caused a greater incorporation of $[^{14}C]$FUra into RNA than did either guanosine or adenosine (4). In Chart 2, data are presented which show the effect of inosine concentration on the uptake of $[^{14}C]$FUra into the acid-soluble pool, the conversion of FUra to FUra nucleotides, and the incorporation into RNA of the N1-S1/FdUrd hepatoma cells. As the concentration of inosine increased, the uptake of FUra into the cells and the percentage of FUra converted to nucleotides likewise increased. The percentage converted to nucleotides reached a maximum of approximately 80%. With this increased conversion to nucleotides, there was a corresponding increase in the incorporation of FUra into RNA.

**Effect of Inosine Concentration of [14C]FUra Incorporation into RNA Species.** Chart 3 shows the effect of inosine concentration on the incorporation of $[^{14}C]$FUra into the various RNA species.
species, as analyzed by sucrose gradient centrifugation. In the absence of inosine, there were barely detectable levels of \(^{[14C]}\text{FUra}\) incorporated into any species of RNA. As the concentration of inosine was increased, there was the corresponding increase in \(^{[14C]}\text{FUra}\) incorporation into RNA. As can be seen, there was significant incorporation of FUra into what appears to be a 45S species, an intermediate species of approximately 32S, and a species in the 4S to 5S region. There was little incorporation of FUra into a species sedimenting as an 18S species. There was considerable accumulation of the 45S species in the presence of FUra and inosine.

In control experiments carried out with \(^{[14C]}\text{Ura}\) plus inosine, 45S, 28S, 18S, and 4S to 5S species of RNA were labeled (data not shown).

Chase experiments were carried out to see if the 45S species which accumulated in the presence of FUra and inosine would eventually lead to labeled 28S and 18S species. As seen in Chart 4, the \(^{[14C]}\text{FUra}\)-labeled RNA did not chase into "mature" 28S and 18S species. There was increased incorporation into the 4S to 5S species which could represent both the 4S and 5S species and the degradation products of the 45S species.

**Effects of the Combination of FUra Plus Inosine on \(^{[14C]}\text{Guanosine}\) Incorporation into RNA.** The incorporation of \(^{[14C]}\text{guanosine}\) into the RNA of the N1-S1/FdUrd cells was studied under the conditions indicated for the \(^{[14C]}\text{FUra}\) experiments. In the control cells, guanosine was incorporated into all of the RNA species (45S, 28S, 18S, and 4S to 5S RNA), as determined by sucrose gradient centrifugation. Inosine alone or FUra alone had no effect on either the total incorporation of guanosine into RNA or into the various RNA species. However, as seen in Chart 5, the combination of FUra plus inosine caused a marked decrease in the incorporation of guanosine into the 18S species, although complete inhibition was not observed. There was an increase in accumulation of the 45S species in the presence of FUra and inosine.

**Effect of the Combination of FUra Plus Inosine on RNA Methylation.** Since the maturation of 45S RNA involves the methylation of the RNA molecule, the effects of inosine, FUra, and the combination of FUra plus inosine on the methylation of RNA were studied. As seen in Chart 6, there was incorporation of methyl groups from \(^{[14C]}\text{methionine}\) into all of the RNA species from the control, inosine-treated, and FUra-treated N1-S1/FdUrd cells. However, in the presence of the inosine-plus-FUra combination, there was little methylation of the 18S species, a decrease in methylation of the 28S species, and an accumulation of the methylated 45S species.

In the FUra-sensitive cells (N1-S1), only FUra was required to prevent the appearance of the methylated 18S species.

**Effect of Preincubation with Inosine on \(^{[14C]}\text{FUra}\) Metabo-
inhibition of L. casei thymidylate synthetase by the presence of FdUMP. In this method, the inhibition of FdUMP could also be determined by this method. Using this method, FdUMP could not be detected in any of the samples. Likewise, there was not an increase in dUMP which would be expected if the thymidylate synthetase reaction were inhibited by FdUMP.

Metabolism of [5-3H]Deoxycytidine in N1-S1 and N1-S1/FdUrd Cells. In an attempt to determine if the combination of inosine plus FURA would alter the thymidylate synthetase reaction in the intact cells, [5-3H]deoxycytidine was incubated with both the N1-S1 and N1-S1/FdUrd cell lines. The acid-soluble fractions were prepared and analyzed in such a manner that the level of dCMP, dUMP, and dTMP (actually as 3H2O) could be determined under the conditions of FURA and inosine alone and in combination in both cell lines. As seen in Table 3, in the sensitive cells (N1-S1), in the presence of FURA alone or FURA plus inosine, there was a marked increase in the total amount of labeled material which accumulated in the acid-soluble fractions. This was due to the marked inhibition of the incorporation of these precursors into DNA (data not shown).

In the sensitive cells, in the absence of drugs, the ratio of dUMP to dTMP (3H2O) was 0.85. In the presence of FURA alone or FURA plus inosine, the ratio of dUMP to dTMP change to 2.64 and 18.5, respectively. The changes in the dUMP:dTMP ratio which were observed were due to the combined increase in dUMP accumulation and decrease in dTMP formation, indicating inhibition at the thymidylate synthetase step. In the resistant cells, in the absence of drugs, the ratio of dUMP to dTMP
Effect of Inosine on \([^{14}\text{C}]\text{FUrd}\) Uptake and Incorporation in RNA. Since inosine caused a marked stimulation of FUrd uptake into the cells and incorporation into RNA, the effect of inosine on \([^{14}\text{C}]\text{FUrd}\) uptake and incorporation into RNA was studied. Inosine had no effect on the uptake of \([^{14}\text{C}]\text{FUrd}\) into the acid-soluble fractions or into the RNA of either the N1-S1 or N1-S1/FdUrd cell lines.

Reversal of Growth Inhibition of the FUrd-Plus-Inosine Combination by Uridine. The N1-S1/FdUrd cells which were originally derived as fluorodeoxyuridine-resistant cells are also cross-resistant to FUrd. As seen in Chart 9, the FUrd alone had only a slight inhibitory effect on the growth of the cells, while inosine alone had no effect. However, the combination of FUrd plus inosine completely prevented growth. The addition of uridine to the FUrd plus inosine combination reversed the growth-inhibitory effects.

DISCUSSION

In order to better understand the metabolism of FUrd leading to its cytotoxic effects, a comparison of the metabolism of FUrd was made in 2 Novikoff hepatoma cell lines. FUrd-sensitive (N1-S1) and FUrd-resistant (N1-S1/FdUrd) cell lines were used in these studies. The N1-S1/FdUrd cell line had been originally defined as being thymidine kinase deficient (11), and we had recently shown that this line was still deficient in thymidine kinase and had an elevated thymidylate synthetase activity (20). It was also shown that the resistant cells failed to convert FUrd to FUrd nucleotides (18). In the present study, we show that the ribonucleotide pools of the FUrd-sensitive and -resistant cells were altered, in that the ratio of UTP to CTP was increased in the resistant cell relative to the sensitive cells. It is possible that part of the resistance to FUrd can be ascribed to this increased level of UTP in the resistant cells, since FUTP competes with UTP for many reactions such as the formation of UDP-glucose, UDP-glucuronate acid, and incorporation into RNA. In the absence of any drugs, the growth rates of the sensitive and resistant Novikoff hepatoma cell lines were essentially the same, with doubling times of 12 and 13 hr, respectively. Further, the cell densities reached at the plateau phase were the same.

Using the approach of Gotto et al. (5), who showed that inosine could stimulate the incorporation of FUrd into nucleic acids, it was possible to reverse the resistance to FUrd by including inosine in the culture medium of the N1-S1/FdUrd cells (4). The presence of inosine caused a marked stimulation of FUrd ribonucleotide formation and incorporation into RNA.

In the current study, experiments were carried out using primarily the FUrd-resistant Novikoff hepatoma cell line to determine if, by “titrating” the system with inosine, it would be possible to determine the relative importance of the incorporation of FUrd into RNA versus the inhibition of thymidylate synthetase by FdUMP.

The presence of inosine in the culture medium led to the increased formation of FUrd nucleotides and incorporation into RNA (Chart 2). The measurement of the ribose-1-P and PRPP levels in the cells after incubation with inosine showed that the ribose-1-P but not the PRPP levels were altered. Inosine, through the reaction catalyzed by purine nucleoside phosphorylase, yields its ribose moiety as ribose-1-P. The ribose-1-P evidently was not utilized for the formation of PRPP via ribose 5-phosphate. This would suggest that the activation of FUrd to nucleotide proceeds via the nucleoside phosphorylase and nucleoside kinase pathway rather than the pyrimidine phosphoribosyltransferase reaction. An interesting but still unresolved question is why the resistant cells need inosine to activate FUrd to the nucleotide level. The cells obviously possess the enzymes necessary for this conversion. It is clear, however, that in the resistant cells inosine had to be present simultaneously with the FUrd to effect the stimulation of formation of FUrd nucleotides and incorporation into RNA.

By increasing the concentration of inosine in the culture medium, the level of \([^{14}\text{C}]\text{FUrd}\) incorporation into RNA was also...
increased. The $^{14}$C-FUra was incorporated into the 45S precursor species, a 32S species, and the 4S to 5S region. There was little incorporation into the 18S species. Attempts to "chase" the 45S precursor into mature 28S and 18S species were not successful. Wilkinson et al. (21) had previously reported that FUra had to be incorporated into RNA in order to inhibit rRNA maturation. These data provide direct evidence to support their conclusion. Alternatively, data were collected which showed that the combination of FUra plus inosine inhibited the incorporation of $^{14}$C guanosine into the 18S rRNA.

With both labeled precursors (FUra or guanosine), there was an apparent accumulation of the 45S precursor RNA. The inhibition of rRNA maturation by the combination of FUra plus inosine appeared to correlate with the alteration in the methylation of the RNA. There was little incorporation of methyl groups into the 18S species, with a lesser inhibition of the methylation of the 28S species and an increase in the labeled 45S species. Whether the lack of methylated 18S species resulted from a faulty RNA which was rapidly degraded, or due to the inhibition of the methylation of that portion of the 45S precursor, is not yet known. Perry (16) had earlier shown that 8-azaguanine inhibited the maturation of the 45S precursor to the 28S, but not to the 18S component, in a manner complementary to the situation described here.

The results show quite clearly that, in the N1-S1/FdUrd cell line, the combination of inosine plus FUra leads to the incorporation of FUra into the RNA of these cells and the RNA metabolism of these cells is greatly altered.

Utilizing the same experimental approach, attempts to measure the levels of dUMP in the acid-soluble portions prepared from cells incubated with $^{14}$C-FUra and various concentrations of inosine were made. Using an HPLC method, FUra, FdUrd, and FdUrd could be easily separated. Analysis of the acid-soluble portions from the FUra plus inosine-treated cells by this technique did not show the presence of FdUrd, although there was a clear increase in FUra levels with the increase in inosine concentration. The control experiments which were carried out showed that between 50 and 100 cpm of authentic FdUrd could be determined which would correspond to 1 to 2 nmol.

Likewise, utilizing the enzymatic method of Myers et al. (12) with thymidylate synthetase from L. casei, FdUMP was not detected in the acid-soluble fraction. In addition, no increase in dUMP levels was observed, as would have been expected if FdUMP were formed and were blocking the thymidylate synthetase step as a result of the FUra plus inosine combination.

To further rule out the thymidylate synthetase step, experiments were carried out in the intact cells (N1-S1 and N1-S1/FdUrd). Since the resistant cells lacked thymidine kinase (11, 20), deoxyuridine could not be utilized as a precursor. Therefore, [5-3H]deoxyxycytidine was used as the precursor to evaluate the effects of these compounds on the thymidylate synthetase step. If [5-3H]deoxyxycytidine is converted to dCMP and then to [5-3H]dUMP, $^3$H2O would be released as a direct measure of the thymidylate synthetase reaction in the intact cell. From the data shown in Table 3, in the sensitive cells, FUra alone or in combination with inosine caused a marked inhibition of $^3$H2O release (inhibition of thymidylate synthetase), with a corresponding accumulation of dUMP due to the inhibition of thymidylate synthetase. In the resistant cells, there was neither the large increase in dUMP accumulation nor the large inhibition of dTMP formation in the presence of the FUra-plus-inosine combination, compared to the inosine alone and the FUra alone.

Ladkin et al. (9) recently reported on the natural variation in sensitivity of various cell lines to FUra. At least one of the factors for the variation in sensitivities to FUra was the extent to which FUra was incorporated into RNA. Nayak et al. (13), with the use of pyrimidine nucleosides, were able to stimulate the incorporation of FUra into the RNA of the mammary and colon tumors, leading to the enhancement of the antitumor properties of FUra.

These present data show quite clearly that in the N1-S1/FdUrd cells, which are resistant to FdUrd because of a thymidine kinase deficiency (19, 20) and also resistant to FUra because of the inability of these cells to "activate" FUra in the absence of inosine, the combination of FUra plus inosine leads to the formation of FUra nucleotides and subsequent incorporation into RNA and no evidence for the formation of FdUMP.
Chart 8. Analysis of snake venom-treated acid-soluble fractions from N1-S1/FdUrd cells by HPLC. Acid-soluble fractions were prepared (14) and were treated with snake venom. The hydrolyzed samples were analyzed by HPLC under conditions identical to those described in Chart 7. The concentrations of FUrA and inosine were as described in Chart 3.
The cytotoxic action of this combination appears to be exclusively due to an "RNA effect" rather than a "DNA effect." Further, these data suggest that uridine should be considered for use as a rescue agent of FUra toxicity.

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Effect of 5-Fluorouracil on RNA Metabolism in Novikoff Hepatoma Cells

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