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The Pharmacology of the Fluoropyrimidines*

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I. Introduction

OVER the past four years there have been major advances in our understanding of the biochemistry and pharmacokinetics of the fluoropyrimidines. They have led to a series of innovative clinical protocols designed to take advantage of the new knowledge. The purpose of this review is to summarize and to attempt to integrate these advances, some of which have called into question long-held views about this class of compounds.

Much of this progress has occurred as a result of a relatively few technical innovations. Foremost among these advances has been the development of high-pressure liquid chromatographic assays for 5-fluorouracil (FUra) and its major metabolites (14, 118, 16) that have greater specificity than the microbiological assays and are faster than the thin-layer chromatographic procedures. A second advance has come through the use of protein binding techniques to study the interaction between thymidylate synthetase and 5-fluorodeoxyuridine monophosphate (FdUMP). This approach, pioneered by the groups working under Santi and Heidelberger, has not only shed light on the details of this inhibitor-enzyme interaction, but has also provided assays of extraordinary sensitivity for FdUMP and thymidylate synthetase. In combination, these two technical advances have illuminated the pharmacology of the fluoropyrimidines, but they have yet to be used to their fullest potential to study the sequence of events that occur in a cell exposed to FUra. These technical advances, however, have stimulated intense interest in the manipulation of intracellular biochemical events after FUra exposure for therapeutic gain. This has been the motivation behind combining FUra with methotrexate, thymidine, or phosphono-N-acetyl-l-aspartic acid (PALA). Each of these combinations gives promise of significantly altering the potency of the toxic/therapeutic ratio of FUra, and they offer tangible evidence for the value of understanding the perturbations FUra causes in intracellular biochemistry.

* The abbreviations used are: SDS, sodium dodecyl sulfate; NMR, nuclear magnetic resonance; UAG, uridylate adenylate guanylate; CAG, cytidylate adenylate guanylate.

II. Metabolism of 5-Fluorouracil

Overwhelming evidence indicates that, unless subject to metabolic activation, FUra is essentially harmless to mammalian cells. Detailed knowledge of this balance between metabolic activation and catabolism is essential to any discussion of the biological effects of these compounds (table 1; fig. 1).

A. Anabolism

There are several pathways by which FUra may be activated. Thymidine phosphorylase has, in addition to its conventional activity, the capacity to catalyze the

transfer of deoxyribose groups from one pyrimidine to another (124). Thus, in the presence of an appropriate deoxyribose donor, this enzyme will catalyze the conversion of FUra to fluorodeoxyuridylate (FdUrd) (11). Conversely, it can facilitate the conversion of administered FdUrd if an appropriate pyrimidine base deoxyribose acceptor is provided. This enzyme appears to be widely distributed, if not ubiquitous, in normal and malignant human cells. Nevertheless, its role in FUra metabolism is unclear. Circulating FdUrd levels are low or undetectable even after administration of toxic levels of FUra (122). In contrast, simultaneous administration of FUra and thymidine, a deoxyribose donor, does lead to significant FdUrd formation, presumably through the action of this enzyme. These results suggest that, under ordinary conditions, this transferase action is limited by the availability of a deoxyribose donor, not FUra concentration, and would be expected to be zero order with respect to FUra. In vitro, FdUrd is appreciably more cytotoxic than FUra. Clinical trials of systemic FdUrd versus FUra, however, have failed to demonstrate much difference between the two either in terms of toxicity or therapeutic efficacy (68, 90). These results are best explained by rapid conversion of FdUrd to FUra through the action of thymidine phosphorylase. Unfortunately, at the time these clinical trials were done, modern assay methodology, which makes simultaneous determination of FUra and FdUrd possible, was not available. This would have been of interest, because we now know that the conversion of FdUrd to FUra could probably be forestalled by coadministration of an alternate deoxyribose donor.

Recent work by Woodman, Sarrif, and Heidelberger (123) has suggested that deoxynucleoside phosphorylase activity may, in fact, reside in three separate enzyme classes. They have shown that, in addition to the specific deoxynucleoside phosphorylase that utilizes FdUrd and

TABLE 1
Metabolism of fluorouracil

1. Thymidine phosphorylase
   dTHd + Pi ⇋ thymine + α-D-deoxyribofuranose-1-phosphate
   FdUrd + Pi ⇋ FUra + α-D-deoxyribofuranose-1-phosphate
2. Uridine phosphorylase
   Urd + Pi ⇋ Ura + α-D-deoxyribofuranose-1-phosphate
   FUrd + Pi ⇋ FUra + α-D-deoxyribofuranose-1-phosphate
3. Uridine-deoxyuridine phosphorylase
   Urd + Pi ⇋ Ura + α-D-ribofuranose-1-phosphate
   dThd + Pi ⇋ thymine + α-D-deoxyribofuranose-1-phosphate
   FUrd + Pi ⇋ FUra + α-D-ribofuranose-1-phosphate
4. Thymidine kinase
   dThd + ATP ⇋ dTMP + ADP
   FdUrd + ATP ⇋ FdUMP + ADP
5. Uridine-cytidine kinase
   Urd + ATP ⇋ UMP + ADP
6. Orotate phosphoribosyltransferase
   Orotic acid + 5-phosphoribosyl-l-pyrophosphate ⇋
   OMP + pyrophosphate
   FUra + 5-phosphoribosyl-l-pyrophosphate ⇋
   FUMP + pyrophosphate
7. Ribonucleotide reductase
   UDP + NADPH ⇋ dUDP
   FUDP + NADPH ⇋ FdUDP
8. Uridylate kinase
   UMP + ATP ⇋ UDP + AMP
   FUMP + ATP ⇋ FUDP + AMP
9. Pyrimidine reductase or dihydrouracil dehydrogenase
   Uracil + NADPH ⇋ β-ureidopropionic acid + NADP
   FUra + NADPH ⇋ 5-f-β-ureidopropionic acid + NADP

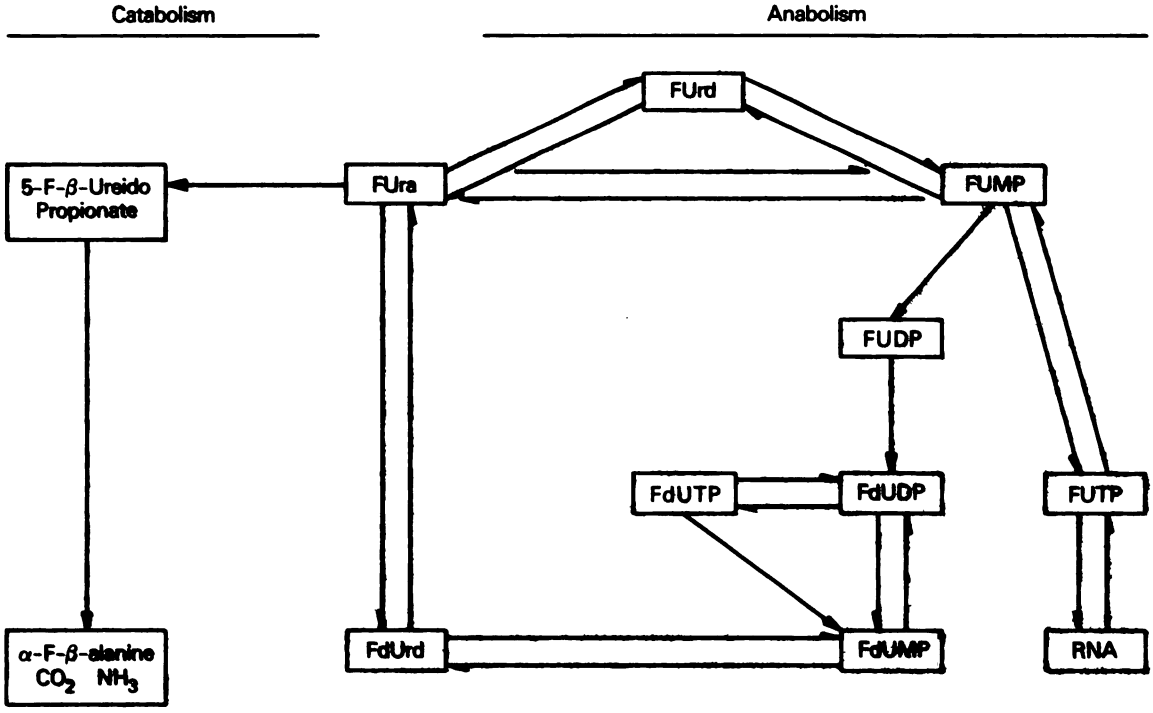

FIG. 1. Enzymatic pathway for 5-fluorouracil (FUra) metabolism.

thymidine but not uridine and the uridine phosphorylase that will use uridine (78), there is a uridine-deoxyuridine phosphorylase able to use uridine, deoxyuridine, and thymidine. The latter can be distinguished readily because it is specifically inhibited by 1-(2′-deoxy-β-D-glucopyranosyl) thymine (125). These authors have shown that the uridine-deoxyuridine phosphorylase predominates in Ehrlich ascites cells and in Novikoff hepatoma cells, whereas the more specific thymidine phosphorylase predominates in mouse liver, human leukocytes, and in HeLa cells. It will be of interest to learn the relative roles of these two enzymes in man. This suggests the possibility of selective blockade of 5-fluorodeoxyuridine cleavage in certain tumor cells, but not host target tissues. It would be of interest to determine what phosphorylase predominates in marrow stem cells and intestinal mucosa crypt cells, the two host-target cells of clinical importance.

FdUrd, either administered or formed as a metabolite of FUra, can act as a substrate for thymidine kinase with resultant conversion to the nucleotide 5-fluorodeoxyuridine monophosphate, FdUMP. As will be discussed, FdUMP is an extremely potent inhibitor of the enzyme thymidylate synthetase. Thus, thymidine phosphorylase and thymidine kinase, acting in concert, represent one enzymatic pathway for FUra activation (fig. 1).

5-FUra can also undergo direct conversion to 5-fluorouridine monophosphate (FUMP) via the action of orotic acid phosphoribosyl transferase. Recent work by Reyes (104) has shown that this enzyme and uracil or pyrimidine phosphoribosyl transferase are probably one and the same and that orotic acid is the preferred substrate (104). 5-FUra is also utilized in preference to uracil in this reaction. This enzyme utilizes 5-phosphoribose-1-pyrophosphate (PRPP), which is a common substrate for this reaction and the first step in purine synthesis, the amidophosphoribosyl transferase reaction. Competition for this common substrate by these various pathways can adversely affect conversion of FUra to FUMP. Similarly, blockade of PRPP utilization for one of the competing pathways can potentially free PRPP for use in FUra activation.

There is an alternate pathway for the conversion of FUra to FUMP. Uridine phosphorylase (78) or uridine-deoxyuridine phosphorylase is capable of catalyzing the formation of 5-fluorouridine from FUra utilizing ribose-1-phosphate. Uridine-cytidine kinase can then complete the conversion to FUMP (1).

The FUMP formed in this reaction may be further phosphorylated to FUDP and FUTP followed by incorporation into RNA. In addition, FUDP may undergo conversion to FdUDP through the action of ribonucleotide reductase. FdUDP is subsequently converted to FdUMP, which can inhibit thymidylate synthetase. Thus, the second and third (fig. 1) pathways for FUra activation are via PRPP transferase or the phosphorylase-kinase coupled to FUMP and hence to FdUMP.

One unsettled question has been why FdUTP was not formed and subsequently incorporated into DNA. Caradonna and Cheng (21) have shown recently that human cells can form FdUTP and that once formed it will act as substrate for alpha DNA polymerase. However, dUTPase actively hydrolyzes FdUTP to FdUMP so rapidly that formation of the triphosphate can only be detected in the presence of excess dUTP. Further, any FdUTP incorporated into DNA can be removed by uracil-DNA glycosylase. It appears that the combination of FdUTP hydrolysis by dUTPase and the action of the glycosylase is sufficient to prevent measurable quantities of FdUTP from reaching the DNA. IdUrd and BdUrd, however, are incorporated into DNA in considerable quantities, and it would be interesting to see how IdUTP and BdUTP differ from FdUTP as substrates for dUTPase and uracil-DNA glycosylase.

Within a given cell line, either one or both of these pathways may be operative. Even where both are present, one or the other may predominate. In murine tumor cell lines, where the data are most complete, there is a general correlation between PRPP transferase activity and tumor response (102), although data recently reported suggest that this is not true for certain cell lines, especially if ribose-1-phosphate or deoxyribose-1-phosphate is available (80). One of the unsolved problems of FUra pharmacokinetics is the relative role of these pathways in a variety of human tumors. Just such a study of a few human tumor cell lines has been reported by Laskin et al. (80). It would be of value to have similar information on a wide range of human malignancies.

B. Catabolism

The catabolism of FUra proceeds via the same pathway as uracil and thymine (17, 18, 55, 16). The pathway and the metabolites formed in the case of uracil, thymine, and FUra are illustrated in table 1 and figure 1. IUrd and BUrd also are ultimately disposed of via this same sequence. However, in the case of IUra and BUra, dehalogenation occurs and, indeed, release of I^{125} from IUra has been used to assay activity of this pathway (100). In mammalian tissue, the rate-limiting step in this pathway is the enzyme dihydrouracil dehydrogenase. While the above information suggests that substrate specificity is broad for this enzyme, one important pyrimidine, cytosine, is not used as a substrate directly, but must first undergo deamination to uracil. Heidelberger and coworkers (90) first suggested that the activity of this catabolic enzyme inversely correlated with response. More recently, Queener et al. (101) have shown that this enzyme correlates directly with the degree of differentiation in tumors and in regnerating liver.

Diazouracil is an irreversible inhibitor of dihydrouracil dehydrogenase and is thus a valuable probe for assessing the quantitative role this enzyme plays in FUra clearance (31, 32). While studies of fluorinated nucleotide formation after FUra administration in the presence and absence of diazouracil have been done in mice, detailed plasma

and tissue pharmacokinetics in man have not been carried out. As will become evident, such an approach might resolve several key questions in FUra pharmacokinetics.

III. Interaction of FdUMP with Thymidylate Synthetase

Of the biological effects of FUra, certainly one of the most dramatic and well described is the interaction of FdUMP with the enzyme thymidylate synthetase. Since much of the currently available information suggests that this interaction may explain the antitumor activity of FUra, an understanding of this reaction has clinical implications as well as being a topic of biochemical interest. In this review, we will focus our discussion, for the most part, on that information necessary to discuss the biological effects of these compounds.

A. Chemistry of the Interaction

Most of the work on the chemical basis for this inhibitor interaction comes from work on the *Lactobacillus casei* enzyme and caution must, therefore, be exercised in extrapolating this information to mammalian tissue. Thymidylate synthetase is composed of two polypeptide chains, each with a molecular weight of approximately 35,000 daltons. Each of the chains has one active site and, indeed, the amino acid sequences of the two chains appear to be identical (84, 85), although, as will be discussed, the kinetic behavior of the two chains may be different. Active site fragments have been isolated by a variety of methods and consist of Ala-Leu-Pro-Pro-Cys-His-Thr-Leu-Tyr (7, 96). Of the four cysteine moieties found in the enzyme, one in this sequence appears to be central to enzymatic activity and FdUMP binding (57, 40, 38, 8, 84). Thus titration with thiol-binding reagents such as iodoacetamide shows loss of both functions after titration of only one thiol group. The resultant carboxymethyl-cysteine has been identified as the one in the center of the above polypeptide. Exposure to the normal competitive substrate, dUMP, prevents iodoacetamide binding and inactivation of this cysteine residue. In enzymes to which FdUMP has bound, this cysteine is no longer found at the active site, but is replaced by an unidentified amino acid that contains sulfur and FdUMP and is presumed to be cysteine-FdUMP. Finally, Raney nickel, which has the unique property of excising sulfur from organic compounds, effectively splits FdUMP from thymidylate synthetase (39).

The mechanism of enzyme catalysis is thought to depend upon nucleophilic attack of the cysteine thiol upon the 5–6 double bond of dUMP resulting in a thioether linkage between cysteine and the 6-position of dUMP (36, 80, 107, 106). This binding in turn is thought to labilize the 5-position hydrogen and facilitate binding of the methylene group of the folic acid cofactor to that site. There follows reduction of the methylene to a methyl group. In the case of FdUMP, this process proceeds to the point at which both enzyme and the folate cofactor are bound, but the resultant complex is very stable compared with the dUMP containing complex and dissociation is thus very slow.

At present, it is not clear to what extent these observations are universal to thymidylate synthetase from differing phylogenetic sources. In fact, while such varying sources as bacteria, phage mammalian cells, and chicken embryo have been used, there are few careful cross species comparisons available on the properties of this enzyme. The potential value of such an analysis can be seen in the elegant work with inhibitors of dihydrofolate reductase (4). That work has led to the identification of antifolates of widely differing properties. There is some evidence that there may also be exploitable differences between thymidylate synthetases from various sources. Historically, the first suggestion of such a difference came from Reyes and Heidelberger (103), who invoked this concept to explain different patterns of enzyme inhibition kinetics reported by groups working with enzyme from bacterial or mammalian sources. This concept received further support when similar differences were noted in enzyme elaborated by T4 as compared to T5 phage (20). Since this early work, however, most of the evidence suggesting species-specific differences in the properties of thymidylate synthetase has come from work with nonfluoropyrimidine inhibitors of this enzyme. Aromatic diamidines such as pentamidine, propamidine, and stilbamidine have been shown to inactivate thymidylate synthetase irreversibly (73). There were, however, major differences between mammalian, bacterial, and protozoan thymidylate sythetases in their sensitivity to this class of compounds and this differential sensitivity was suggested to be the basis for the effectiveness of these agents as antiprotozoal drugs.

Human thymidylate synthetase recently has been purified to homogeneity and partially characterized (78, 46). It also appears to be composed of two 33,000 dalton fragments and binds FdUMP into a tight ternary complex with $N^{5,10}$methylene tetrahydrofolic acid. As with the bacterial enzyme, methotrexate antagonizes inactivation of the enzyme by FdUMP. There appear to be significant differences in amino acid composition between this enzyme and the thoroughly characterized *L. casei* thymidylate synthetase, particularly in the apparent number of cysteine residues. Given the importance of cysteine in the mechanism of catalysis and in the interaction with FdUMP, such differences deserve more careful examination.

The existence of tissue-specific differences in thymidylate synthetases would open the possibility of analogs tailored to tumors arising from certain tissues. The best evidence that such tissue-specific differences do occur remains the observation by Barfknecht et al. (5) that 5-iodoacetamidomethyl-2′-deoxyuridine-5′-phosphate is nearly 3 times more effective in inhibiting the enzyme from Ehrlich ascites tumor cells than it was in inhibiting enzyme from thymus.

From these considerations it is apparent that there are both tissue and species specific differences in the sensitivity of thymidylate synthetase to a variety of inhibitors and this is a topic that warrants further study.

B. Kinetics of the Interaction

Kinetic analysis of high-affinity enzyme inhibitors represents a problem of considerable magnitude. Many of the assumptions underlying conventional Lineweaver-Burk analysis of inhibition are violated by high-affinity inhibitors. This problem has been extensively discussed recently by several authors, each of whom presents alternative approaches to this problem (89, 61). The root cause of much of this complexity arises from trying to determine the details of enzyme-inhibitor interactions by their effects on substrate-enzyme interactions. In fact, analysis of high-affinity inhibitors is often dramatically simplified if one studies directly the enzyme-inhibitor interaction. The fluoropyrimidines represent a classic case study of these analytic problems and their appropriate solution.

1. Methods of study. For years, this field was immersed in an unproductive debate on whether FdUMP was a competitive inhibitor and whether preincubation rendered the interaction noncompetitive. This discussion masked the real questions about the nature of the inhibitor-enzyme interaction. The breakthrough came when it was realized that this interaction could be directly studied by examining the binding of labeled FdUMP to thymidylate synthetase. This insight, which occurred nearly simultaneously in the groups working under Santi (107) and Heidelberger (79), has allowed rapid progress in our understanding of both the mechanism and kinetics of the FdUMP-thymidylate synthetase interaction. There are other techniques that have also been used to study this enzyme-inhibitor interaction directly, each of which has provided additional important information. Of these, calorimety (6), fluorescence quenching (111), and circular dichroism (47) have been of particular value. One practical outgrowth of these studies has been the development, by two groups, of sensitive competitive protein binding assays for FdUMP similar in design to that previously established for methotrexate. These competitive binding assays are far more rapid, sensitive, and precise than the previous thin-layer and enzyme-inhibition assays. As has been pointed out by one of these groups (88), this technique also provides the investigator with a highly sensitive assay for tissue thymidylate synthetase levels.

2. Equilibrium, association, and dissociation reactions. One of the earliest observations reported by those using the protein binding techniques was that the enzyme-inhibitor complex appeared to be essentially irreversible when the denatured enzyme was examined (79, 107). It was thus reported that the complex would not dissociate after exposure to SDS or after acid precipitation of the protein. Subsequent workers have been able to digest the enzyme with proteases and, as mentioned previously, were able to isolate a peptide fragment with FdUMP attached (8, 113). These studies stand in marked contrast to others that have repeatedly shown dissociation of FdUMP from native enzyme (108). This unusual increase in apparent stability of the binding with protein denaturation had led Danenberg (36) to conclude that dissociation itself was catalyzed by the native enzyme, and to propose a mechanism based upon the Mannich reaction. As he pointed out, this reaction mechanism has certain properties relevant to the FdUMP-thymidylate synthetase interaction.

One of the advantages of the ligand-binding approach is that it can be modified to allow direct measurement of the association and dissociation reaction between an enzyme and its inhibitor. Often this can provide clearer information about the inhibitor-enzyme interaction than can be obtained from equilibrium binding studies. When the interaction between inhibitor and enzyme becomes more complex than a simple association-dissociation reaction, analysis either by enzyme inhibition or equilibrium binding techniques is difficult. For example, Scatchard plot interpretation becomes ambiguous when one is dealing with more than one distinct class of binding site or with cooperativity between binding sites. In such a situation, full resolution of the interaction may require analysis of the association and dissociation reactions. One of the advantages of ligand-binding techniques is that they can be modified to allow direct measurement of the association and dissociation reaction. This information, when supplemented with physical techniques such as NMR and circular dichroism, will often provide a clearer picture of the interaction. The binding of FdUMP with thymidylate synthetase is an example of such a situation. Scatchard plot analysis of the binding yields a concave up-curve that is consistent with either negative cooperativity or two distinct binding site classes (91). Previous work had shown that the enzyme was composed of two polypeptide chains, each capable of binding 1 mole of FdUMP. As we have already pointed out, it has been shown that the amino acid composition and sequences of the two units are identical (84, 85). Dunlap and coworkers developed a polyacrylamide gel technique that allowed separation of thymidylate synthetase from enzyme bound to 1 or 2 moles of FdUMP per mole of enzyme (3). With this technique, they showed that binding of the first mole of FdUMP occurred much more avidly than the second (47). Others have subsequently confirmed this observation (35, 37). These results, when combined with the Scatchhard analysis and amino acid sequence data, suggest that the enzyme sites are identical but exhibit negative cooperativity. A recent study by Danenberg and Danenberg indicates that such a simple model is probably not adequate (35, 37). As mentioned previously, titration of thiol groups in the enzyme with reagents such as N-ethylmaleimide reveal a single reactive site whose titration results in a loss of

enzyme activity; this thiol, as we discussed, is more likely cysteine. These results suggested that the cysteine in the second of the two active sites was unavailable for titration. Danenberg et al. (35, 37) reexamined this phenomenon and found that binding of the first FdUMP opened the second active site to titration by these thiol reagents. As they pointed out, this suggests that the second site is sterically hindered until conformational changes associated with binding to the first site make it available. Once open for binding, however, the second site is still not equivalent to the first in its avidity for FdUMP.

3. Interaction with deoxyuridylate. In vivo, the interaction of FdUMP with thymidylate synthetase does not occur in isolation but in a complex milieu in which the concentrations of the folate cofactor, dUMP, and enzyme all vary with time and in response to drug exposure. Study of these factors is much less advanced than is the study of the binding of FdUMP to the enzyme under conditions outlined above. The potential importance of these factors was first suggested by Bosch et al. (13), who showed reversal of FUra inhibition of DNA synthesis by deoxyuridine, presumably via augmentation of intracellular dUMP concentration. This matter received more detailed examination by Myers et al. (94). These authors showed that the rate at which FdUMP inactivated thymidylate synthetase was dependent upon the dUMP concentration; higher dUMP levels were able to lessen the rate of thymidylate synthetase inactivation dramatically, presumably via active site occupancy. This is a mechanism similar to that which had been proposed for the effect of acetylcholine on inactivation of acetylcholinesterase by diisopropyl fluorophosphate (2). This interaction is described by the equation:

$$\log 1/\alpha = (2.3\ k_a\ [I]\ t)/(1 + [S]/K_m) \qquad (1)$$

where k_a is the bimolecular rate constant; K_m is the Michaelis constant of S; α is the fraction of initial enzyme activity remaining; t is the time elapsed after the inhibitor is added; and I and S are the inhibitor and substrate concentrations. This equation predicts that the rate of enzyme inactivation should drop rapidly as the concentration of substrate exceeds the K_m. Also, the rate of inactivation should be first order with respect to time for any combination of substrate and inhibitor concentrations. These authors also showed that dUMP pools expand in certain tissues such as bone marrow after exposure to FUra and suggested that dUMP accumulation played a role in recovery of that tissue. Similar accumulation of dUMP in cells exposed to FUra has subsequently been reported by numerous workers (115, 71, 15, 76), but the correlation with response has been much less apparent. These studies suggest that a much more comprehensive examination of the intracellular events would be of value. There are a number of difficulties with the early studies of the effect of dUMP accumulation on 5-FUra response. First, the above studies on the interaction of dUMP, FdUMP, and thymidylate synthetase were done by using enzyme activity as the measured parameter. Second, equation 1 assumes irreversible binding. We now know that the binding to native enzyme is reversible. It is obvious that this interaction needs to be reexamined by using ligand-binding techniques and the model used for analysis should include terms for FdUMP-thymidylate synthetase dissociation, although quantitatively this may be minor under most conditions. Nonequivalence of the two sites likewise needs to be considered. Third, the newer assays for FdUMP, dUMP, and thymidylate synthetase are much more precise and rapid. Thus, it is apparent that the relationship between these moieties and response should be reexamined. Preliminary work in this direction has already been published (14). Ideally, such studies require accurate measurement, not only of free FdUMP, dUMP, and thymidylate synthetase, but also of the pertinent folate pools, $N^{5,10}$methylene tetrahydrofolic acid at the least. In addition, the FdUMP-thymidylate synthetase ternary complex should be measured in some way, by using specific antibody to the protein (81), Raney nickel release of bound FdUMP (39), release by boiling (118), or protein-bound radioactivity after administration of labeled FUra. Such information is of more than just theoretical interest; it would provide a logical framework for understanding some of the biological effects of such combinations as FUra-thymidine, FUra-methotrexate, and FUra-PALA, for example. Beginning efforts in this direction have been made by Washtien and Santi (118), Moran et al. (88), Bowen et al. (14), Laskin et al. (80), and Cadman et al. (16).

IV. Effects on RNA Synthesis, Processing, and Function

In most cell lines, FUra appears to be rapidly converted to FUMP and incorporated into the various RNA species. Messenger RNAs in which FUMP replaces UMP have been isolated and their function has been studied by a number of investigators (64, 119). Miscoding has been observed, the best example of this being in the *Escherichia coli* amber mutant (105). There FUra replaces uracil in the amber codon resulting in the UAG code being translated as CAG. The end result is reversion of the amber mutant. In HeLa cells, Gleason et al. (60) have shown that FUra can be converted to 5-fluorocytosine, which is incorporated into mRNA in place of cytosine and is then misread as uracil. Carrico and Glazer (22) have shown that FUra-modified polyadenylic acid RNA translates at 2.5 times the rate of unmodified poly(A)RNA. This stimulation appeared to be limited to elongation of the nascent polypeptide chains and did not involve initiation or release. While the mechanism of this effect remains obscure, it indicates that the effect of FUra on translation is more complicated than simple miscoding.

Fluorouracil has additional, major effects on ribosomal RNA. Work by several groups now has confirmed that while preribosomal RNA synthesis is not inhibited, the

subsequent processing and maturation of the RNA is markedly affected (22, 23, 64, 120). Two alterations have been noted; first there is a drop in the production of the 18S and 28S species, and second there is a drop in RNA methylation thought to be essential to RNA maturation. Fluorouracil thus joins a list of drugs with effects of possible biological importance on ribosomal RNA production. Anthracyclines such as Aclacinomycin A have a specific effect on preribosomal RNA synthesis (49): Combination of such agents with FUra offers the opportunity for sequential blockade of ribosome production.

V. Relative Importance of RNA Effects versus Thymidylate Synthetase Inhibition

This problem has been a source of controversy for years and has recently been subjected to extensive reexamination. The difficulties are in part technical; it has been much easier to study and quantify the inhibition of thymidylate synthetase by FdUMP than it has been to study the more subtle effects on RNA synthesis and function. Recently, two additional problems have come to light that may help explain the confusing results that have been reported. First, Plagemann (97) and others (77) have shown that there are probably two ribonucleotide pools—one cytoplasmic the other nuclear in location. Further difficulties arise because the rates at which these two pools equilibrate with one another vary markedly from one mammalian cell line to another. At this point, one can only speculate what effects this phenomenon has on studies of FUra phosphorylation and incorporation into RNA. These studies do emphasize once again that a great deal of caution is needed in interpreting effects on incorporation of radiolabeled precursors into RNA and DNA. Unfortunately, such experimental techniques are frequently interpreted without appropriate caution. The second important observation has been made by Washtien and Santi (118). These authors have shown that thymidylate synthetase-bound FdUMP can represent a significant proportion of the total FdUMP formed in a cell. They also showed that this bound pool could be released by heating the tissue sample at 65°C for 15 min and the FdUMP was then assayed. They argued convincingly that only by assaying the total FdUMP pool rather than just free FdUMP can one get an accurate estimate of the magnitude of FdUMP formation by a given cell line or tissue. Furthermore, free FdUMP does not give an estimate of the degree to which thymidylate synthetase has been inhibited. It would seem that measurement of bound FdUMP and active thymidylate synthetase would be more relevant than the free FdUMP pool. Sensitive assays of the latter are available. As will become evident, this point has been generally ignored.

One common approach to this problem has been to examine sensitive versus resistant tumor cell lines and to see if differences in sensitivity correlated with either FdUMP formation or RNA effects. The earliest efforts in this field were by Reyes and Reyes and Hall (104, 102), who showed that phosphoryation of FUra via PRPP transferase was the major determinant of FUra sensitivity. Such phosphorylation could, of course, lead to both FdUMP and FUMP formation and does not serve to distinguish between the two mechanisms of action. Subsequently, several workers have found a correlation between accumulation of free FdUMP and sensitivity. This correlation has taken two forms. Certain authors have simply observed a correlation between free FdUMP concentration and response. Others have observed a correlation between the rate of FdUMP clearance and response. The variation in FdUMP clearance was first noted by Myers et al. (93, 94) in a comparison of bone marrow versus duodenal mucosa, wherein they observed rapid clearance from duodenal mucosa and slow clearance from bone marrow. These observations have been extended to tumor cells by Klubes et al. (76). When they compared the resistant Walker 256 tumor with the more sensitive L1210 tumor, they found peak levels greater in Walker 256 but this tumor exhibited much more rapid clearance of FdUMP and inhibition of DNA synthesis was similarly abbreviated in this tumor. In contrast to these studies, there are a series of papers in which no correlation of any kind was found between FdUMP formation and response, the most comprehensive of which was a study by Houghton et al. (70). These authors examined both free FdUMP formation and incorporation of the fluoropyrimidine into RNA of the gastrointestinal mucosa after exposure to FUra, fluorouridine, and FdUrd. They observed no correlation between free FdUMP levels at 1 hour after each of these different agents and their respective abilities to inhibit thymidine incorporation. They did, however, observe a remarkable correlation between RNA fluoropyrimidine concentration, thymidine incorporation, and toxicity. There are three potential problems with this study. First, free rather than total FdUMP was measured. Perhaps more importantly, only the 1-hour time point was examined. Also, whole animal toxicity rather than gastrointestinal toxicity was used for the purpose of comparison. As we have discussed, it is possible that persistence of FdUMP may be as important as peak levels. Also, the kinetics of FdUMP formation and clearance may differ significantly for the various fluoropyrimidines, especially since fluorouridine and FdUrd may have entirely different pathways of enzymatic activation. In spite of these minor objections, this work has a number of interesting implications. Based on the findings of Houghton et al., one would have predicted that any agent that increased FUra incorporation into RNA might result in increased gastrointestinal toxicity. This prediction has certainly been borne out in the trials of FUra-thymidine combination in which toxicity was seen at doses of from one-half to two-thirds of the conventional i.v. bolus therapy.

A second paper pertinent to this question resulted from a detailed analysis of a FUra-resistant Novikoff hepatoma cell line (33). Under the usual conditions of

culture, this line was not an active phosphorylator of FUra. Inclusion of inosine in the culture medium resulted in restoration of sensitivity, phosphorylation of FUra, and its subsequent incorporation into RNA. This resulted in the expected decrease in 18S ribosomal RNA formation and RNA methylation. In contrast, free FdUMP could not be detected in these cells whether grown in the presence of FUra alone or in the presence of FUra plus inosine. As with the previous study, the major problem in interpretation is that free rather than total FdUMP was measured.

The interaction of FUra and methotrexate has been examined extensively (10, 15, 75, 115, 116). The covalent binding of FdUMP to thymidylate synthetase requires the availability of reduced folate cofactor, the production of which is blocked by methotrexate. While methotrexate can substitute for $N^{5,10}$methylene tetrahydrofolate, it does not permit the same high affinity binding. Thus, methotrexate-induced decline in $N^{5,10}$methylene tetrahydrofolic acid concentration should antagonize the FdUMP-dependent inhibition of thymidylate synthetase. However, as has been pointed out by Cadman et al. (16), methotrexate blocks purine biosynthesis and the PRPP not utilized there would become available for the conversion of FUra to FUMP via the action of phosphoribosyl transferase with subsequent incorporation into RNA. Cadman et al. (16) documented just such a sequence of events in L1210 cells treated with methotrexate followed by FUra. Because this sequence also was synergistic in terms of tumor cell kill, these authors have argued that the synergism must be based on increased RNA effects. This argument is based on the assumption that these methotrexate-treated cells were in fact, deficient in reduced folate. This was not demonstrated. The experimental technique involved cloning the treated cells in soft agar after drug exposure. Under these conditions, methotrexate is likely to diffuse out of the cells rapidly and the block in folate metabolism should cease. FdUMP, on the other hand, does not readily diffuse across cell membranes and, as we have discussed, may have a long intracellular half life. It is thus indeed possible for covalent binding of FdUMP to proceed once the cells are plated into soft agar as the methotrexate-induced block in folate metabolism is reversed. The observed synergism then might as well be due to the almost 50-fold increase in FdUMP formation that the authors observed after methotrexate exposure. Again, these authors also measured only free FdUMP. The mechanism by which methotrexate exposure leads to increased FdUMP formation was further clarified by Elford et al. (50), who have shown that methotrexate causes a 360% to 400% increase in ribonucleotide reductase, the enzyme responsible for the conversion of FUDP to FdUDP. Furthermore, this increased activity resulted from new enzyme synthesis rather than from increase in the activity of enzyme already present. From this, one can surmise that first the reduction in purine biosynthesis leads to increased availability of PRPP, which in turn allows increased formation of FUMP, FUDP, and FUTP. The increased levels of FUDP plus increased levels of ribonucleotide reductase in turn lead to increased formation of FdUDP and FdUMP. This sequence of events requires the presence of PRPP transferase and ribonucleotide reductase, and that levels of PRPP be limiting. For example, should a cell lack phosphoribosyl transferase, but contain thymidine phosphorylase and thymidine kinase, methotrexate might have little or no effect on phosphorylation to either FUMP or FdUMP. In addition, Bowen, White, and Goldman (15) have suggested that the antagonism between methotrexate and FUra may be more complex. They point out that FUra will reduce cellular requirements for reduced folates by lessening its consumption in the thymidylate synthetase reaction and decreasing the need for purine synthesis. However, the capacity of dihydrofolate reductase to reduce dihydrofolate to tetrahydrofolate is already so great that excess intracellular methotrexate is required to block this pathway. The net effect of FUra pretreatment, then, is to increase significantly the intracellular methotrexate required to achieve a limiting concentration of reduced folate. This whole picture is further complicated by the fact that methotrexate pretreatment leads to a dramatic increase in thymidylate synthetase levels in rat liver and human lymphoblasts, thus rendering blockade of thymidylate production more difficult (12, 26). From all of this work, it is apparent that methotrexate can be synergistic or antagonistic in its interaction with FUra, depending on the sequence used and the metabolic machinery present in the target cell, and it is not at all clear that this drug interaction provides evidence in support of RNA as the site of action for FUra.

The interaction of thymidine and FUra recently has been the focus of intense scrutiny and the effects that have been observed have been attributed to increased incorporation of FUra into RNA. These results have been one of the major stimuli behind the current reexamination of the importance of the RNA effects. It has now been well documented that high concentrations of thymidine lead to increased incorporation of FUra into RNA in many cell lines (23, 50, 54, 95). In addition, there appears to be a correlation between this increased incorporation of FUra into RNA and enhancement of antitumor efficacy in several experimental systems (54, 86, 95, 106, 121). These observations are counter to other in vitro studies that have clearly shown either no enhancement or partial rescue of tumor cells by thymidine (92, 117). An understanding of this apparent conflict requires some discussion of the complex biochemical events likely to follow combined exposure to FUra and thymidine. In cells with adequate levels of thymidine kinase, exposure to thymidine will lead to augmented dTTP pools. This, in turn, will lead to feedback inhibition of ribonucleotide reductase and thus lessen the formation of FdUMP from FUMP through the diphosphate intermediates. In addition, activation of FdUrd through thymidine kinase will

be blocked by end-product inhibition by the high TTP pools. The net effect would be to lessen activation of FUra to FdUMP. In addition, the "thymidineless state," thought to be caused by FdUMP, will be relieved. Thymidine is thus well suited as an agent to prevent the formation of FdUMP and most of it consequences. For cells that possess the capacity to form FUMP through the action of PRPP transferase, then, intracellular FUra becomes available almost entirely for conversion to FUMP and subsequent incorporation into RNA. In a tissue culture system, this scenario can be used to explain either rescue or increased toxicity. In cells lacking the capacity to form FUMP, thymidine will simply prevent any effects due to FdUMP formation. In a cell line with the capacity to form FUMP and sensitive to the alterations in RNA function, thymidine might lead to increased cell kill. These events have yet to be fully documented. In vivo, the situation becomes more complex. Thymidine appears to be catabolized via dihydrouracil dehydrogenase in a fashion analagous to 5-FUra. Competition for this common catabolic pathway is thought to underlie the dramatic decrease in FUra clearance in man that follows thymidine administration (74, 121). In addition, as mentioned earlier, Houghton et al. (70) have reported a good correlation between incorporation of fluoropyrimidines into RNA and toxicity in the mouse. These results do provide a reasonable explanation for the increased toxicity seen following the FUra-thymidine combination as compared to FUra alone. At present, it is not clear whether the increased toxicity seen in man with the thymidine-FUra combination reflects the impact of this delayed clearance or increased incorporation itno RNA. Table 2 summarizes the range of possible effects thymidine can have on FUra pharmacology.

While there is no question that extensive incorporation of FUra into RNA occurs, this does not necessarily lead to biologically significant alterations in RNA function. While the studies of Carrico and Glazer (22, 23) and others have taken careful inventory of the changes in RNA function that occur, direct evidence that this results in cell death is lacking. RNA does turn over and may thus act as a slow release depot for FUMP over time. A fraction of this FUMP may be converted to FdUMP. Thus, manipulations that increase the substitution of FUra for Ura in RNA may also serve to support the FdUMP pool for far longer than would otherwise occur. Thus, it is possible that the effect of FUra incorporated into RNA is not a lethal lesion in RNA function, but rather prolongation of FdUMP-mediated blockade of thymidylate synthetase. The proper test of this hypothesis would be to measure depression in thymidylate synthetase activity or amount of FdUMP bound to the enzyme over time after FUra alone and in combination with one of the drugs known to enhance incorporation of FUra into RNA.

TABLE 2
Thymidine effects on FUra pharmacology

1. Increased thymidine pools resulting in:
   a. Reversal of "thymidineless state";
   b. Feedback inhibition of ribonucleotide reductase, which lessens FdUMP formation from FUMP.
2. Slows catabolism via competition for dihydrouracil dehydrogenase thus lessening the rate of FUra clearance.
3. Increased levels of FUMP secondary to 1 and 2 above, which in turn lead to increased incorporation of fluorinated nucleotides into RNA.
4. A probable increase in FdUrd formation from FUra via the action of the ubiquitous phosphorylases with thymidine acting as a deoxyribose donor.
5. As a result of RNA turnover, the enlarged pool of fluorinated nucleotide containing RNA may act as a source for the prolonged release of FUMP and thus formation of FdUMP. Such an action might lead to prolonged release of FUMP, formation of FdUMP, and prolonged suppression of thymidylate synthetase.

In summary, the whole question of the biological consequences of FUra incorporation into RNA is central to our understanding, not only of FUra action, but also its interaction with thymidine, methotrexate, inosine, and other compounds as well (33, 34). While it now seems likely that incorporation of FUra into RNA plays a role in the cytotoxicity of this drug for certain cell lines, proper evaluation of this effect, as compared to FdUMP formation, is rendered difficult by the incompleteness of the published studies. There have been common problems such as evaluation of total versus free FdUMP and problems associated with the use of radiolabeled precursors. The means for solution of these problems are now available. The recent development of high-pressure liquid chromatographic techniques allows facile measurement of pyrimidine pools (14, 16, 118). In addition, the highly sensitive specific ligand binding techniques pioneered by Santi and coworkers (108, 118), Heidelberger and coworkers (79, 88), and confirmed by our group (91) allow more complete characterization of FdUMP generation and its effects on thymidylate synthetase. The comprehensive studies by Carrico and Glazer (22, 23) provide a model of how to quantify RNA effects. Full resolution of this problem is most likely to occur when all of these techniques are applied simultaneously to a series of sensitive versus resistant cell lines.

VI. Sequential Inhibition of the de Novo Synthesis of Thymidylate

Sartorelli and Creasey (109) have discussed in detail the theory of combination chemotherapy. From a biochemical point of view, one of the most interesting aspects is the use of sequential inhibitors of a key biosynthetic pathway. The observation by Myers et al. (94) that dUMP accumulation may play a role in reversing the effect of FUra on a cell suggests that sequential blockade of the pyrimidine pathway might block dUMP accumulation and enhance the cytotoxicity of FUra. This leads naturally to the use of inhibitors of pyrimidine biosynthesis that act on earlier steps than does FUra. The list of potential agents is long and includes hydroxyurea, an inhibitor of ribonucleotide reductase, pyrazofurin, and 6-

azauridine, both inhibitors of orotidylate decarboxylase, and PALA (30), an inhibitor of aspartate transcarbamylase. Several of these agents have been used in experimental chemotherapy systems aimed at exploiting the concept of sequential blockade. However, there are a number of considerations that complicate this approach. Hydroxyurea, because it blocks ribonucleotide reductase, would lessen conversion of FUMP to FdUMP. Pyrazofurin, allopurinol, and 6-azauridine would lead to accumulation of orotic acid; the orotic acid thus formed may compete with FUra for orotic acid phosphoribosyl transferase and thus lessen conversion of FUra to FUMP. PALA, because it acts so early in the de novo pathway, overcomes most of these problems. In addition, by preventing orotic acid synthesis, this compound should maximize FUMP formation via the orotic acid PRPP transferase.

These concepts are supported by recent work reported by Schwartz and Handschumacher (110) on the interaction between 4-hydroxy-pyrazolopyrimidine (HPP) (allopurinol) and FUra. These authors have shown that HPP antagonizes 5-FU cytotoxicity in a number of cell lines because it leads to accumulation of orotate, which, in turn, competes with 5-FU for the orotate phosphoribosyltransferase pathway.

Perhaps the major problem with this whole approach is that it is difficult to avoid simultaneously increasing host tissue toxicity as well as antitumor effect. For example, there is evidence that suggests that dUMP accumulation may be critical for bone marrow recovery after FUra. In that case, simultaneous sequential inhibition of the pyrimidine pathway might be expected to increase bone marrow toxicity.

VII. Selective Effect of Fluorocytosine on Fungal Cells

After the discovery of a drug with significant biological activity such as FUra, it is common to see the development of an extensive analog synthesis program. Such an analog synthesis program can occasionally lead to the production of agents with important activity in clinical situations entirely divorced from that of the prototype. The synthesis of fluorocytosine follows this pattern. After FUra had proved to have activity against a variety of murine and human tumors, Heidelberger and his collaborators synthesized a variety of fluorinated pyrimidines including fluorocytosine. When these were tested for biological activity, fluorocytosine was found to be essentially inactive against mammalian cells (48, 67). This agent was then tested against a variety of bacterial and fungal strains. In these tests, fluorocytosine did exhibit activity against several fungal strains, including *Candida albicans* and *Cryptococcus neoformans* (62). Fluorocytosine was then subjected to clinical trial as a single agent in the treatment of these two fungal diseases. While responses were noted, they were not associated with cure of the fungal disease and were in general short-lived responses. However, in vitro studies suggested that fluorocytosine could be effectively combined with amphotericin B, an active but extremely toxic antifungal agent (87). A recent randomized clinical trial of the fluorocytosine-ampotericin B combination versus amphotericin B alone has confirmed the value of this combination (9). The combination proved to be significantly less toxic than amphotericin alone, presumably because the synergism of the combination permitted the use of a lower amphotericin dosage level.

The biochemical basis for this selective antifungal action is complex. The transport of pyrimidines into *C. albicans*, for example, is controlled by a permease system (98). Candida strains that have been studied commonly have permeases for uracil and cytosine but lack permeases for thymine or thymidine (63). These cells thus are dependent on de novo thymidine synthesis and do not have the capacity of mammalian cells to scavange preformed thymidine from their environment. This property suggests that they would be susceptible to blockade of thymidylate synthesis. In addition, many strains of *C. albicans* and *C. neoformans* possess a cytosine deaminase that avidly deaminates fluorocytosine to FUra. Presence of this deaminase has been shown to correlate with sensitivity to fluorocytosine and its deletion correlates with the development of resistance (72). Extensive incorporation of the labeled FUra thus formed into RNA of sensitive strains has been shown (99). This has been interpreted as reflecting synthesis of FUTP from fluorocytosine via FUra and FUMP. A majority of the workers in this field appear to favor this incorporation into RNA as the major mechanism of antifungal activity (59, 99). These workers had not, however, adequately examined the possibility of FdUMP formation and its consequences. In a recent paper from our group, Diasio, Bennett, and Myers (45) have reexamined the antifungal activity of this drug and its relationship to FdUMP formation in a fluorocytosine-sensitive strain of *C. albicans*. The logarithmically growing cultures ceased growth within 1 hour of addition of fluorocytosine to the culture media. This cessation of growth corresponded temporally with the disappearance of thymidylate synthetase from the fungal cells and the appearance of free FdUMP. These results suggested that rapid production of FdUMP did occur and that the amount produced was in excess of that required for titration of most of the thymidylate synthetase available. In parallel with earlier reports from this laboratory (94), dUMP pools expanded with time after fluorocytosine in a manner similar to that seen in mammalian cells after exposure to FUra. Ultimately, fungal cell growth resumed in spite of the continued presence of FdUMP. These results all suggest that the response of *C. albicans* to FdUMP formation is similar to that of mammalian cells. Thus, FdUMP formation in a cell dependent on de novo thymidylate

synthesis is as reasonable an explanation for the antifungal activity of this agent as is incorporation of the fluoropyrimidines into RNA.

Finally, the subject of the cytotoxicity of fluorocytosine has been reexamined recently. In the clinical trials where fluorocytosine has been evaluated, moderate to severe bone marrow suppression was noted. Recently, Diasio et al. (44) have shown that FUra can be bound in serum of such patients. The levels were, in some cases, in excess of 10 μM, a concentration sufficient to explain the myelosuppression observed.

VIII. Pharmacokinetics of 5-Fluorouracil

There is now a considerable body of literature on the pharmacokinetics of FUra administered via a variety of routes and schedules. This information recently has been subjected to detailed pharmacokinetic analysis by Collins et al. (29) with the physiological modeling techniques that this group has applied to a variety of drugs including cytosine arabinoside (41) and methotrexate (43). In this review, we will discuss the general conclusions of that analysis.

A. Methodological Considerations

The assay of FUra in biological samples was for years accomplished either by microbiological assay or thin-layer chromatography. These assays were relatively insensitive, time-consuming, and, in the case of the microbiological assay, of uncertain specificity. More recently, assays based upon gas chromatography with and without mass spectroscopy or high-pressure liquid chromatography have been developed (table 3). While not necessarily either simpler or more rapid than the older techniques, they do have the advantage of greater sensitivity and inherent specificity. Nevertheless, as will become apparent, results obtained via these different techniques yielded a fairly consistent, if anomalous, picture of FUra pharmacokinetics.

A more serious problem in the analysis of FUra pharmacokinetics is one that generally exists for drugs rapidly cleared from plasma. For such drugs, the value for the area under the concentration versus time curve, essential for analysis of bioavailability and clearance calculations, is determined to a large extent by the early time points obtained. In many of the published studies, too few samples were obtained during the early time periods. In addition, errors or uncertainty in measurement of the time interval between dose administration and sample collection can have more serious consequences for a rapidly cleared drug such as FUra. Such problems almost certainly contribute to the variability seen in published FUra data. These considerations are of greater importance for i.v. bolus pharmacokinetics than for steady state infusions.

B. Bolus versus Continuous Intravenous Infusion of 5-Fluorouracil

The conventional method of administering FUra is to give a single i.v. bolus injection of FUra at a dose of 10 to 15 mg/kg once a month or daily times five once a month. As a result, the most comprehensive pharmacokinetic data are available for i.v. bolus administration. These results, as summarized in the paper of Collins et al. (29), are presented in table 3. In total, studies have been published on 82 patients and reveal a T½ of between 8.2 and 20 min, a volume of distribution of 8 to 54 liters, and clearance values of 0.6 to 1.9 liters/min. In contrast, the pharmacokinetics of constant i.v. infusions have been reported by only four groups, each done at a different infusion rate ranging from 10 to 175 mg/kg/day. The results, again presented in table 3, differ significantly from the i.v. bolus data in implying a much larger clearance value, one approaching the cardiac output. These results have raised two questions. How can the clearance of a drug equal or exceed the cardiac output? Is the clearance of FUra saturable?

TABLE 3
Pharmacokinetics of fluorouracil

Study	Bolus or Infusion	Dose	No. of Patients	T½ (min) (bolus)	Steady State Concentration (infusion)	Total Body Clearance (l/min)	Vd (l)
Cano et al. (19)	Bolus	750–1000 mg	9	12		0.8	
Christophidis et al. (25)	Bolus	11 mg/kg	12	19		0.8	25
Clarkson et al. (27)	Bolus	15 mg/kg	3	12			
Cohen et al (28)	Bolus	15 mg/kg	7	10		0.6	8
Finch et al. (52)	Bolus	500 mg	11	8.2		1.0	13
Finn and Sadee (53)	Bolus	15 mg/kg	3	12		1.5	25
Garrett et al. (58)	Bolus	500 mg	3	12		1.5	25
		1000 mg	3	8.5		1.7	20
MacMillan et al. (83)	Bolus	11 mg/kg	8	11		1.3	18
Sitar et al. (112)	Bolus	11 mg/kg	16	20		1.9	54
Clarkson et al. (27)	Infusion	10 mg/kg/day	2		0.8	4.0	
Ensminger et al. (51)	Infusion	134 mg/kg/day	8		71.0	0.94	
Garrett et al. (58)	Infusion	175 mg/kg/day	3		15.0	3.6	
Hillcoat et al. (69)	Infusion	30 mg/kg/day	27		1.6	7.0	

For most organs, clearance is limited by organ blood flow, which is, of course, only a fraction of the cardiac output. This is certainly true for the liver, traditionally thought to be the major site of FUra catabolism (24). First, dihydrouracil dehydrogenase is present in its highest concentration in this organ (101). This enzyme, as was discussed earlier, catalyzes the probable rate-limiting step in pyrimidine catabolism. Second, 80% of C^{14}-labeled FUra appears as respiratory carbon dioxide, the expected end product of the catabolic pathway of which dihydrouracil dehydrogenase is the limiting step (66). Third, clearance values calculated from i.v. bolus pharmacokinetics approximate liver blood flow, which ranges between 1 to 1.5 liters/min. Ensminger et al. (51) have recently estimated hepatic extraction at 50%. Hepatic clearance rates, thus, would be expected to range between 0.5 to 0.8 liters/min, significantly lower than most of the clearance values for continuous infusion FUra. In addition, dihydrouracil dehydrogenase activity is found in other tissues. In the rat, where extensive data are available, the comparative levels reported, in μmoles/gm/hour, are 2 for liver, 0.9 for gastrointestinal mucosa, 0.7 for spleen, 0.6 for kidney, 0.4 for brain, and 0.2 for skeletal muscle (101). In each of these tissues, however, clearance will also be limited by total blood flow and extractions of certainly less than 100%. Catabolism at these sites cannot, therefore, explain clearance values that equal or exceed cardiac output. There are two tissues in which clearance values can equal or exceed cardiac output; these are blood and lung. In these two, there is no evidence to suggest catabolism of FUra by either plasma or red blood cells. In pharmacokinetic studies conducted on FUra, we have found no loss in measurable drug levels after prolonged contact with either blood or plasma. While pulmonary metabolism has not been specifically studied, the consequences of such metabolism have received thorough analysis by Collins et al. (29). These authors have shown that, even if the lung were the only site of drug clearance, it is possible for that clearance to exceed cardiac output. In this situation, the total body clearance, Cl_{TB}, is described by the equation:

$$Cl_{TB} = Q\,E_p/(1 - E_p) \qquad (2)$$

where Q is the cardiac output and E_p is the pulmonary extraction. Inspection of equation 2 shows that pulmonary extraction has only to reach 0.5 for Cl_{TB} to equal the cardiac output. However, both hepatic and renal clearance of FUra have been described and metabolism at other sites is implied by the distribution of dihydrouracil dehydrogenase. In that situation, CL_{TB} is defined by equation 3:

$$Cl_{TB} = \Sigma_i(Q_i\,E_i) + Q\,E_p/(1 - E_p) \qquad (3)$$

where $\Sigma_i\,(Q_i\,E_i)$ is the sum of the clearances at organs i with blood flow of Q_i and extraction of E_i. In this setting, pulmonary extraction can be significantly less than 0.5 and Cl_{TB} will still exceed cardiac output. Thus, it is possible for clearance to exceed the cardiac output, but this implies clearance of FUra in the blood or lung or both.

The data for i.v. bolus and continuous infusion administration show a general pattern of decreasing clearance with increasing FUra dosage. This observation is consistant with a nonlinear or saturable clearance process. This is unusual because the published i.v. bolus disappearance curves do not show the convex log plasma concentration-time curve one expects from a saturable process. Again, Collins et al. (29) have shown that it is possible to construct a saturable two-compartment model that satisfactorily predicts disappearance kinetics after i.v. bolus and constant infusion drug administration and does not yield a convex disappearance curve. In applying their model to the published data, Collins et al. (29) found the best fit of a K_m for the saturable process was assigned a value of 15 μM. It is of interest that dihydrouracil dehydrogenase also is responsible for the degradation of thymine (17, 18, 55, 56). Thus, competition for this rate-limiting step provides a facile explanation for the observed saturability of 5-fluorouracil clearance. In addition, competition for this site provides a reasonable explanation for the ability of high thymidine dosages to decrease FUra clearance (74, 121, 122). Additional evidence suggesting the importance of dihydrouracil dehydrogenase comes from studies with diazouracil. This drug, which is an irreversible inhibitor of dihydrouracil dehydrogenase, increases the conversion of FUra to nucleotides and increases both toxicity and antitumor activity (33, 34). These results suggest that under normal circumstances, clearance via dihydrouracil dehydrogenase significantly limits bioavailability of FUra.

C. Pharmacokinetics of Oral 5-Fluorouracil

In order to analyze the pharmacokinetics of oral FUra, one must consider absorption across the gastrointestinal mucosa, catabolism within the mucosa itself, and clearance during passage through the liver in addition to factors affecting systemic clearance. There is virtually no data available on the gut absorption process for FUra. Catabolism by gut mucosa has been generally ignored in the analysis of oral absorption of FUra, although gut mucosa contains the second highest level of dihydrouracil dehydrogenase and specific gut clearance has been measured by at least one group (65). Thus, appropriate modeling of oral pharmacokinetics of this drug will require provision for first-pass effects in both the gut and liver, in addition to an absorption term.

The one observation that stands out most prominently on review of the published literature on oral absorption of FUra is the large variability in peak levels attained and in overall bioavailability (14, 25, 28, 52, 53, 58). Collins et al. (29), in their analysis of the published literature, estimated that the first-order rate constant for oral absorption varied over greater than a two log range. As discussed above, their model did not contain terms

for gut metabolism; the results are, however, sufficient to suggest that the oral route is too unpredictable to be of clinical value.

D. Pharmacology of Intraperitoneal 5-Fluorouracil

It has long been a matter of common clinical practice to use intracavitary FUra or FdUrd to control malignant pleural or peritoneal effusions. Clarkson et al. published, in 1964 (27), pharmacokinetic data on FUra and FdUrd administered to a few patients and reported control of ascites in some patients. We have recently had the opportunity to reexamine the pharmacology of i.p. FUra in greater detail as an adjunct to a phase I clinical trial of FUra administered via peritoneal dialysis (115). The design of this trial was based on the recent analysis of the principles governing i.p. pharmacokinetics published by Dedrick et al. (42). In this trial, dose-limiting toxicity consisted of pancytopenia and mucositis and was seen at dialysis concentrations of 4.5 to 5.0 mM administered for 8 consecutive 4-hour exchanges. Disappearance of FUra from the peritoneum was a first order function with an average peritoneal clearance of 14 ml/min. Within 4 hours, 82% of the drug was absorbed. Throughout the dialysis, plasma levels were much lower than peritoneal fluid levels, with mean 4-hour peritoneal fluid drug levels averaging nearly 300 times the simultaneously measured plasma levels. Total body clearances ranged from 0.9 to 15 liters/min, values which are remarkably similar to those observed with continuous systemic i.v. infusions of FUra. As with the studies discussed earlier, there was a trend for clearances to decrease with increasing FUra concentration, consistent with saturable or nonlinear pharmacokinetics. As a result of the apparent saturation of FUra clearance, there was a rapid increase in both bioavailability and toxicity over a narrow dosage range; severe toxicity was rarely seen at dosage levels of 4 mM, while life-threatening toxicity was seen in all patients treated in this fashion at 5 mM.

These results obtained with FUra show excellent agreement with the general principles governing the pharmacokinetics of i.p. drug administration as recently reviewed by Dedrick et al. (41). The clearance of hydrophilic drugs such as FUra from the peritoneum is governed by molecular size: The value of 14 ml/min is what would have been predicted for FUra based on the behavior of such substances as glucose, urea, creatinine, and inulin. The ratio of plasma to peritoneal drug levels is determined by the ratio of their clearances; again, the results with FUra were consistent with that equation. As a result, the model proposed by Dedrick et al. (41) provides the first detailed pharmacokinetic framework to guide the intracavitary administration of FUra.

IX. Summary and Conclusions

Since the initial synthesis of FUra in the 1950s, this compound has established itself as having a broad spectrum of antitumor activity. Analogs of this compound have proved to be valuable antifungal and antiviral agents. In spite of this progress, the possibilities for further improvement in the use of this class of agents is as great as at any point in the past. This is the case, by and large, because of the strides that have been made recently in our understanding of the complex biochemistry and pharmacology of these agents. As a result, we now have a much clearer understanding of the changes these agents cause in cells and how these changes affect combination of the fluoropyrimidines with other agents such as thymidine and methotrexate. It is in this particular area that there is the greatest need for further study and it is here also where the potential for clinical benefits is the highest.

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