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Vaccinia virus-infected animal cells have been used to study the interactions between the replication of deoxyribonucleic acid (DNA) and the biosynthesis of its nucleotide precursors. Some antimetabolites that inhibit DNA replication have as their targets enzymes of nucleotide biosynthesis. Furthermore, the disruption of nucleotide metabolism can alter the fidelity of DNA replication.

The isolation of viral mutants resistant to the nucleoside analogue arabinosyl cytosine has shown that resistance to certain drugs is associated with altered fidelity of replication of DNA. Furthermore, single mutations can cause altered sensitivity to several compounds. On the other hand, selection of apparent revertants indicates that several sites can be involved in drug resistance and replication fidelity.

Vaccinia virus has previously been shown to code for enzymes of DNA precursor metabolism. However, attempts to isolate mutants resistant to drugs that target thymidylate synthase and dihydrofolate reductase were not successful.

In fact, stimulation of host cell DNA synthesis makes vaccinia virus extremely sensitive to the effects of the folate analogue methotrexate.

Taken together these observations suggest that despite its cytoplasmic site of DNA replication and its large coding capacity, vaccinia virus depends, at least in part, on host cell enzymes to supply deoxynucleoside triphosphates for DNA replication.

Studies on DNA Precursor Metabolism in Vaccinia Virus-Infected Mammalian Cells

By

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Chapter I. INTRODUCTION

The purpose of the studies described in this thesis was to understand interactions between the metabolism of deoxyribonucleic acid (DNA) precursors and the replication of DNA. The faithful replication of DNA is critical to the preservation of species; on the other hand, mutation contributes to evolution. Among the determinants of replication fidelity are the relative concentrations of the deoxyribonucleotide precursors of DNA (Weymouth et al., 1978; Fersht, 1979; Hibner and Alberts, 1980; Meuth et al., 1979a, 1979b; Weinberg et al., 1981, 1985). Observations during the last decade have suggested that the replication of eukaryotic DNA and the biosynthesis of its precursors are closely coordinated (Reddy and Pardee,1980,1983; Reddy, 1982; Noguchi et al., 1983; Rode et al,1980,1985; Navalgund et al., 1980). While the interrelationships between DNA replication and precursor synthesis in eukaryotes remain unclear, alterations in metabolism of DNA precursors have been shown to affect mutation frequency (Meuth et al.,1979a, 1979b; Weinberg et al., 1981, 1985; Kunz, 1983; Roguska and Gudas, 1984; Chu et al., 1984), and precursor metabolism is the site of action of some anti-metabolites that inhibit DNA

replication (Kisliuk and Brown, 1977; Danenberg, 1977; Meuth et al., 1979b; Weinberg et al., 1981, 1985; Schimke, 1984; Schimke et al., 1985). I have used drugs that interfere with DNA precursor metabolism in vaccinia virus-infected cells to clarify the effects of perturbing nucleotide metabolism, specifically with respect to fidelity of DNA replication and the mechanism of action of these drugs.

Studies in prokaryotes have provided evidence for physically and kinetically associated multienzyme complexes of the intricately regulated enzymes of DNA precursor biosynthesis (Mathews, 1985; Chiu et al., 1982). These complexes channel DNA precursors to create effective dNTP concentrations at replication sites that are greater than the average concentrations determined by pool measurements (Mathews and Sinha, 1982). The situation in eukaryotes is more controversial (Chiba et al., 1984; reviewed in Mathews and Slabaugh, 1986).

When extracts of bacteriophage T4-infected Escherichia coli cells are analyzed on sucrose gradients, several activities of nucleotide metabolism co-sediment. These activities include ribonucleotide reductase, thymidylate synthase, nucleoside diphosphate kinase, and deoxycytidylate hydroxymethylase but not DNA polymerase (Reddy et al., 1977; Reddy and Mathews, 1978). In eukaryotes, there is evidence for specificity of interactions among enzymes of DNA metabolism. By human DNA-mediated transformation of mutant

mouse cells deficient in thymidylate synthase, Ayusawa et al. (1983) restored thymidylate synthase activity to the mouse cells; however, no thymidylate synthase activity was detected in a rapidly sedimenting aggregate from sucrose gradients that contained DNA polymerase α and thymidine kinase. In contrast, the aggregate from the wild-type mouse cells did contain thymidylate synthase activity.

Reddy and co-workers have also attempted to demonstrate a physical association among DNA precursor enzymes in eukaryotic cells. They report that, during S phase, enzymes of DNA precursor metabolism migrate to the nucleus and form multi-enzyme complexes (Reddy and Pardee, 1980). Through several purification steps, DNA polymerase and thymidine kinase remain associated with a large aggregate, although no enzymes of *de novo* deoxynucleotide biosynthesis have been confirmed as part of this aggregate (Noguchi et al, 1983). Reddy and co-workers have reported kinetic coupling of enzymes of DNA precursor biosynthesis: permeabilized CHEF/18 hamster cells (Reddy and Pardee, 1982) and extracts from CHEF/18 cells (Noguchi et al, 1983) preferentially incorporate a labeled ribonucleotide (cytidine diphosphate, CDP) into DNA without dilution by an excess of unlabeled deoxycytidine triphosphate (dCTP), the substrate for DNA polymerase.

Recently, two groups (Engström et al., 1984; Leeds et al., 1985) have demonstrated that ribonucleotide reductase,

one of the enzymes of the multi-enzyme complex of Reddy and co-workers (Reddy and Pardee, 1980; Noguchi et al., 1983) is located in the cytoplasm, suggesting cytoplasmic dNTP biosynthesis. Species differences might account for the discrepancy between the studies of Reddy et al. and the other groups. However, in studies done to confirm the conclusions of Reddy and Pardee (1982) with respect to the incorporation of CDP into DNA, Spyrou and Reichard (1983) have reported that in permeabilized CHEF/18 cells, as well as in 3T6 cells, the labeled CDP is incorporated into ribonucleic acid (RNA) and not into DNA. Thus, there remain questions as to the physical and kinetic associations of the enzymes of DNA precursor metabolism.

Nucleotides are differentially concentrated in cellular compartments (Fridland, 1973; Skoog and Bjursell,1974; Bestwick et al.,1982). In addition, Bestwick and Mathews (1982) have shown that nuclear and mitochondrial DNA are labeled at different rates by exogenous precursors. These results suggest that the replication forks do not receive nucleotides from a single cellular pool.

A mammalian system accessible to genetic and biochemical approaches, analogous to bacteriophage T4 (which has been used in the prokaryotic studies referred to above [Mathews, 1985; Chiu et al., 1982]), would help answer questions about the physical and kinetic organization of DNA precursor enzymes. Studies with such a system could also

help define the effect of DNA precursor metabolism on fidelity of DNA replication and clarify the mechanism of action of therapeutically useful drugs that exploit the special nature of DNA precursors.

The DNA genome of vaccinia virus has the capacity to code for a large number of genes. There are about one hundred early genes distributed throughout the vaccinia genome (Belle Isle et al., 1981). Some have been localized but only a few identified (Moss, 1985). About one half of the viral genome is transcribed prior to DNA replication (Kaverin et al., 1975). Vaccinia virus replicates in the cytoplasm (McFadden and Dales, 1982; Moss, 1985). Therefore, the precursors for viral DNA biosynthesis must be supplied to a site in a different cellular compartment from the site of cellular DNA biosynthesis. As suggested above, however, enzymes of DNA precursor biosynthesis are located in the cytoplasm. Leeds et al. (1985) have proposed that the compartmentation of dNTPs arises from the partitioning of enzymes that metabolize nucleotides (for example, ribonucleotide reductase is cytoplasmic [Engström et al, 1984; Leeds et al., 1985] while DNA polymerase α is located in the nucleus [Weissbach, 1977; Bensch et al., 1982]).

Does vaccinia virus control the synthesis of DNA precursors during infection? Is the virus-infected cell a suitable model for studying perturbations of mammalian precursor metabolism that lead to altered mutation rates and

to cell death?

Deoxyribonucleotide Biosynthesis in Mammalian Cells

The proximal precursors of DNA synthesis are 2'-deoxyribonucleoside 5'-triphosphates (dNTPs): deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP), and thymidine triphosphate (dTTP). All known DNA polymerases use 2'-deoxyribonucleoside 5'-triphosphates in preference to deoxynucleosides of lower phosphorylation levels. No known DNA polymerase uses 2'-deoxynucleoside 3'-triphosphates and synthetic pathways for such nucleotides have not been described. All DNA polymerases require a free 3'-hydroxyl group on the deoxyribose of the primer terminus and require a 5'-triphosphate on the nucleotide substrate for chain elongation (Kornberg, 1980).

In contrast to other macromolecular precursors, the dNTPs are specialized molecules whose metabolic roles are limited to DNA synthesis, which occurs at a limited number of intracellular sites during a defined portion of the cell cycle, and perhaps to glycoprotein biosynthesis by way of nucleotide sugars (Mathews, 1985). By contrast ribonucleoside triphosphates (rNTPs,NTPs) play several important roles in cellular metabolism other than utilization for the synthesis of RNA.

The dNTPs are supplied primarily by *de novo* synthesis in pathways that do not include formation of their component free bases or nucleosides (Figure 1.1). Salvage pathways recycle free bases and nucleosides produced by breakdown of nucleic acid and use nucleosides and bases from exogenous sources. In Figure 1.1, the salvage of two nucleosides, deoxyuridine (UdR) and thymidine (TdR), to their respective nucleoside monophosphates is illustrated. The salvage pathways have been exploited therapeutically and experimentally (see below).

The first step in committing nucleotides to their roles as DNA precursors is the reduction of the four ribonucleoside diphosphates (rNDPs or NDPs) to deoxyribonucleoside diphosphates (dNDPs) by ribonucleotide reductase [indicated by asterisks (*) in Figure 1.1] (Thelander and Reichard, 1979; Kornberg, 1980). The four dNDP products of ribonucleotide reductase are DNA precursors; for deoxyuridine diphosphate (dUDP) the pathway to DNA involves conversion to a thymine nucleotide, as uracil-containing nucleotides are not normal constituents of DNA. Both dUDP and dCDP are precursors to the thymine nucleotides, as they are metabolized to dUMP, which is the substrate for thymidylate synthase (Jackson, 1978).

A study of the biosynthesis of DNA precursors suggests the peculiar sensitivity of the pathway leading to the synthesis of thymidine triphosphate (TTP) (see Figure 1.1).

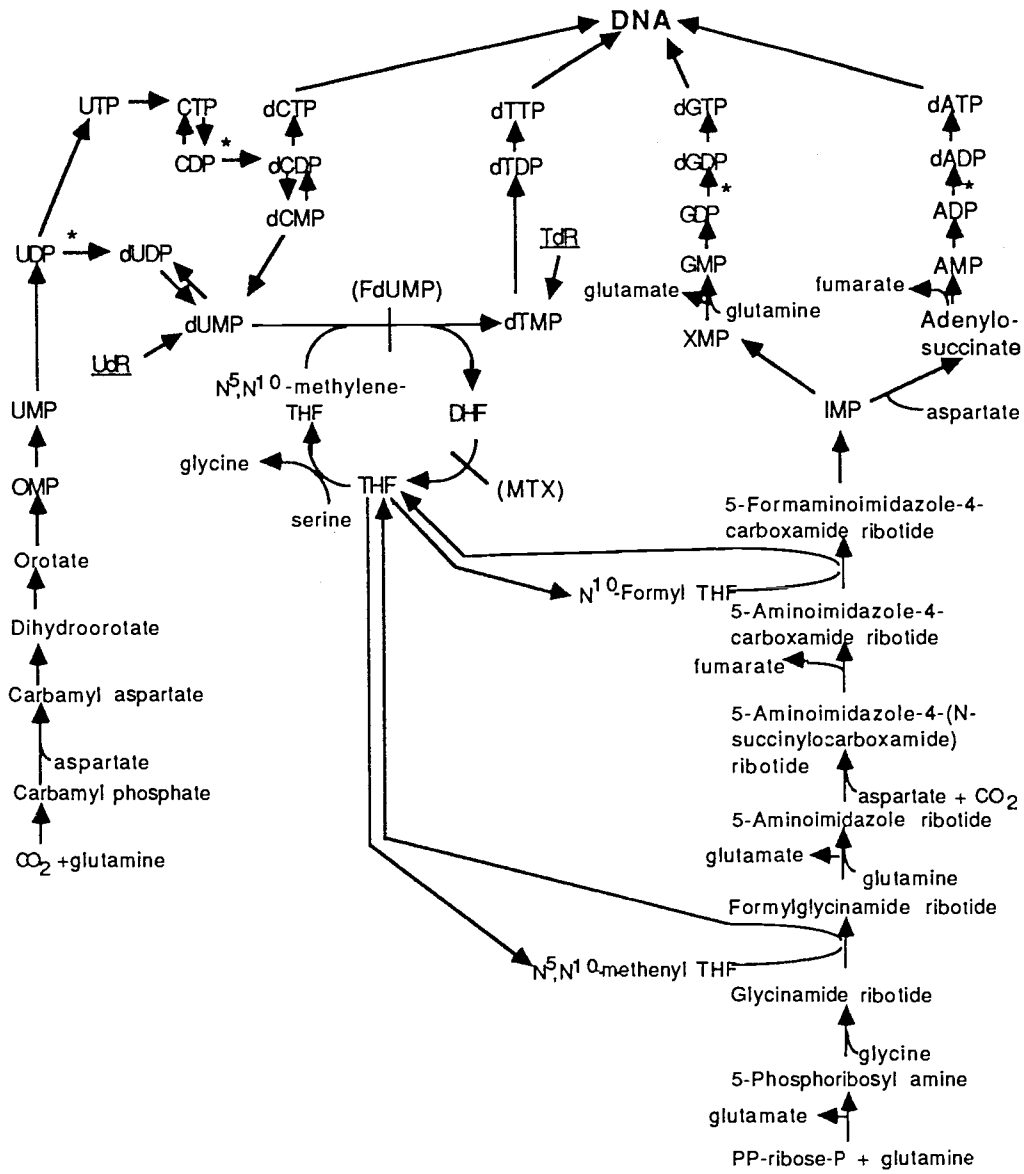

Figure 1.1. Pathways of de novo biosynthesis of DNA precursors. Salvage of deoxyuridine (UdR) and thymidine (TdR) and sites of action of two inhibitors [(MTX) and (FdUMP)] are also indicated. The reactions labeled with an asterisk (*) are catalyzed by ribonucleotide reductase. See text for explanation.

Among the DNA precursors, only thymine nucleotides do not normally occur as ribonucleotides. The other deoxynucleoside diphosphates are products of ribonucleotide reductase (Thelander and Reichard, 1979). Thymidylate (dTMP) is formed by the methylation of deoxyuridylate (dUMP) in a reaction that oxidizes tetrahydrofolate to dihydrofolate (Friedkin and Roberts, 1956; Danenberg, 1977). The reduction of ribonucleoside diphosphates and the synthesis of thymidylate therefore represent reactions specific for deoxyribonucleotide metabolism.

Since the dNTPs are specialized, it is not surprising that levels of dNTPs are lower than those of rNTPs (Garrett and Santi, 1979; Bestwick et al., 1982) and that nuclear dNTP levels increase during S phase, the period of cellular DNA replication (Skoog and Bjursell, 1974; Leeds et al., 1985). Cohen and Barner (1954) reported that depriving thymine auxotrophic bacteria of thymidine leads to "unbalanced growth" and cell death, which they termed "thymineless death." Subsequently, this observation has been exploited to develop agents that are selectively toxic to proliferating cells, those cells that must synthesize DNA (see below).

The enzyme that incorporates the dNTPs into DNA is a DNA-dependent DNA polymerase. Mammalian cells possess several distinct species. The primary replicative polymerase is polymerase α, which has been localized to the

nucleus (Weissbach, 1977; Bensch et al., 1982). In growing cells, DNA polymerase α is responsible for at least 85% of DNA polymerase activity, while in quiescent cells it accounts for only about 5% (Kornberg, 1980). The *in vitro* rate of polymerase α DNA synthesis has been reported at 30 nucleotides per second (Detera et al, 1981), which approaches the *in vivo* rate of fork movement, measured at 50-100 nucleotides per second (Collins et al, 1980).

Drugs as experimental agents

DNA synthesis can be interrupted or deranged by agents or conditions that alter the metabolism of the nucleotide precursors. For example, Hopkins and Goodman (1980) demonstrated that base analogs could cause mutations by perturbations of normal dNTP pools as well as by predicted mispairing with analog bases. As mentioned above, Cohen and Barner (1954) demonstrated that starving thymine auxotrophs resulted in bacterial cell death. Initially this observation was somewhat puzzling as the depletion of thymine would seem only to result in an inability of the cell to make DNA during replication, in contrast to depletion of other metabolites that are involved in the maintenance of cellular integrity and generation of energy. The death of the cell was attributed to "unbalanced growth," as RNA and protein metabolism were apparently not inhibited.

I have used antimetabolites and exogenous DNA precursors in the studies described in this thesis. Drugs that alter nucleotide metabolism are desirable experimental tools. Firstly, they can derange DNA precursor metabolism by affecting specific, or a limited number of, targets. Such manipulations of metabolism can give clues to the usual pathways and regulation of the biosynthesis of DNA precursors. Mutants are particularly useful tools for such investigations, but they are often difficult to select in mammalian cells. In the absence of mutants, drugs have been useful experimental tools, being used as selective agents for mutations that alter nucleotide metabolism. DNA precursor metabolism is closely connected to the replication of DNA and influences the fidelity with which DNA is copied. "Normal" DNA precursors (nucleosides and bases) and their analogues have become useful experimental tools (e.g., HAT medium; gpt, MTX-resistant DHFR as dominant selectable markers). Finally, because they target cells or virus that are synthesizing DNA (but not terminally differentiated cells), DNA precursors and their analogues are valuable therapeutic agents. Therefore, the drugs themselves are of important, and they contribute to our understanding of the crucial processes of DNA precursor biosynthesis and regulation.

On the other hand, adding compounds that derange a biological system may result in a variety of non-specific

effects. For example, the folate analogue methotrexate (MTX)(see Appendix 1) is a specific and potent inhibitor of dihydrofolate reductase (DHFR) (Nichol and Welch, 1950; Osborn et al., 1958) If cells are prevented from recycling folates, those pathways (including purine, thymidylate, and amino acid) that require folate cofactors as carbon donors are disrupted. The target of the drug is clear, but discerning the effects on cells is very complicated, as the extensive literature on MTX demonstrates. Schimke and co-workers (Alt et al., 1978; Schimke, 1984; Schimke et al., 1985) have clarified one aspect of MTX action; they have demonstrated that multiplication of the gene for dihydrofolate reductase can lead to an increase in the synthesis of the enzyme, thereby increasing the ability of the cell to reduce oxidized folate co-factors. Another observation confirms DHFR as the target of MTX (Haber et al., 1981). Mutant cells have been isolated with alterations in the dihydrofolate reductase gene. The protein encoded by the defective gene has a lowered affinity for the drug and confers on a cell the ability to grow in concentrations of MTX that are toxic to normal cells.

Another observation with respect to MTX toxicity is that the reaction that converts deoxyuridylate (dUMP) to thymidylate (dTMP) is the only known reaction in mammlian cells in which the folate cofactor is oxidized from tetrahydrofolate to dihydrofolate (Figure 1.1). Only in

cells synthesizing DNA and, therefore, requiring thymine nucleotides is DHFR essential for reducing folate co-factors (Moran et al., 1979; Chapter 3 of this thesis). Toxicity, then, is dependent on active DNA precursor biosynthesis. When there is oxidation of tetrahydrofolate to dihydrofolate, as mentioned above, purine and amino acid metabolism, as well as thymidylate biosynthesis, are disrupted.

Tetrahydrofolate is required for thymidylate biosynthesis (Friedkin and Roberts, 1956) and measurements of dTTP pools confirm that MTX treatment, in general, depletes thymine nucleotides (Adams et al., 1971; Tattersall and Harrap, 1973; Tattersall et al., 1974; Fridland, 1974; Skoog et al., 1976; Jackson, 1978; Kinahan et al., 1979; Goulian et al., 1980a; Taylor et al., 1982; Appendix A of this thesis). There is evidence, however, that the disturbed supply of purines is, at least under some conditions, the cause of cell death (Hryniuk, 1972; Hryniuk, 1975; Hryniuk et al., 1975). Goulian and co-workers have suggested that increased levels of dUTP and misincorporation of uracil into DNA causes repeated repair and ultimately lethal damage to the genome (Goulian et al., 1980b), and they suggest that uracil misincorporation is the cause of "thymineless" death (Cohen and Barner, 1954). Studies on the biochemical and cell cycle effects of MTX have supplied plentiful data on the effects of the drug (see, for example,

Taylor and Tattersall, 1981; Taylor et al., 1982). The studies on the action of the drug are potentially useful in designing more effective therapeutic regimes that target cancer cells while sparing normal cells. The studies on the ultimate toxic event, that is, the studies that look for specific events distant from the binding of MTX to DHFR, are less useful for an understanding of nucleotide metabolism. Woodcock and Cooper (1981) have suggested that DNA synthesis inhibitors, with a variety of targets, can ultimately kill cells by the same mechanism (see also Schimke et al., 1985).

MTX has been used in studies described below in attempts to understand some aspects of nucleotide metabolism in vaccinia virus-infected cells (Chapter 3) and to clarify the mechanism by which MTX is toxic to cells (Appenidix A).

The experiments described in this thesis have made use of several other drugs as well. The lesson of the use of drugs has been this: when the drug is chosen to answer a specific question or to achieve a specific end (for example, selection of a mutant) and the study is properly designed, the drug is a powerful tool. More often (and as has been my experience), experiments making use of antimetabolites can be difficult to interpret because of the complex metabolic effects of the drugs. Below, I briefly mention the drugs used in my experiments.

The nucleoside arabinosyl cytosine (araC) is a potent antineoplastic (Clarkson et al., 1975) and antiviral (North

and Cohen, 1979; North, 1983, 1984; see Chapter 2) agent . AraC is phosphorylated to araCTP and inhibits DNA synthesis (see Chapter 2 Introduction). It has been successfully used as a selective agent for isolating mutants with alterations in nucleotide metabolism. Studies with these mutants have provided information on the regulation of nucleotide biosynthesis and the consequences of nucleotide pool imbalance on fidelity of DNA replication (Meuth et al., 1979a, 1979b, 1982; Robert de Saint Vincent et al., 1980; Robert de Saint Vincent and Buttin, 1980; Weinberg et al., 1981, 1985; Trudel et al., 1984; Meuth, 1984). AraC is an inhibitor of vaccinia virus DNA synthesis. I have isolated mutants resistant to its effects (see Chapter 2).

Aphidicolin is an inhibitor of replicative DNA polymerases. It is a tetracyclic diterpenoid that has been considered a specific inhibitor of DNA polymerase α (see Huberman, 1981), it is also an inhibitor of viral DNA polymerases (Frank et al., 1984; Defilippes, 1984). The mechanism of inhibition is not clear , but competition with dNTPs has been shown in some systems (Frank et al., 1984). Some mutants with altered sensitivity to aphidicolin have elevated mutation rates (Liu et al., 1983, 1984; see Chapter 2).

The antiviral phosphonoacetic acid (PAA) is a potent inhibitor of herpesvirus DNA polymerase (Overby et al., 1974; Honess and Watson, 1977; Honess et al., 1984). PAA-

resistant mutants are easily isolated (Honess and Watson, 1977; Honess et al., 1984). While the vaccinia virus DNA polymerase is somewhat less sensitive to the drug, it is more sensitive than cellular DNA polymerase α, and selection of virus resistant to PAA was useful in identifying vaccinia virus-induced DNA polymerase as a virus-encoded enzyme (Moss and Cooper, 1982; Sridhar and Condit, 1983). Some PAA-resistant mutants have been shown to encode DNA polymerases with altered replication fidelity (Hall et al., 1984, 1985; Chapter 2).

Fluordeoxyuridine (FUdR) is phosphorylated to fluorodeoxyuridylate (FdUMP), an inhibitor of thymidylate synthase (Danenberg, 1977). In some cells, FdUMP is an important metabolite of 5-fluorouracil (FU), a base widely used in cancer therapy. Two mechanisms of killing by FU have been proposed. FU can be phosphorylated to FUTP, which is incorporated into cellular RNA and disrupts RNA maturation and function (Wilkinson and Pitot, 1973; Carrico and Glazer, 1979; Myers, 1981). Alternatively, inhibition of thymidylate synthase occurs through the formation of a ternary complex containing thymidylate synthase, FdUMP, and the methylene tetrahydrofolate cofactor (see Figure 1.1) (Santi et al., 1974; Danenberg, 1977). Inhibition of thymidylate synthase leads to a decrease in levels of dTTP and an increase in levels of dUTP with incorporation of uracil into DNA (Ingraham et al., 1982). Incorporation of

FU into DNA may play a role in cytotoxicity (Major et al., 1982; Cheng and Nakayama, 1983). The relative importance of these two mechanisms seems to differ among different cells and tumors (Maybaum et al., 1980). In some cell lines treatment with FUdR leads to an increase in the levels of thymidylate synthase (Washtien, 1984).

I have studied the effects of these drugs on vaccinia virus replication, and I have attempted to isolate viral mutants resistant to the effects of the drugs. The results of my studies are described below.

Chapter II. DRUG-RESISTANT MUTANTS OF VACCINIA VIRUS WITH ALTERED REPLICATION FIDELITY

INTRODUCTION

Vaccinia virus, a large, double-stranded DNA-containing virus, replicates in the cytoplasm (Moss et al, 1983; Moss, 1985; McFadden and Dales, 1982). The large coding capacity of the virus and the cytoplasmic site of viral DNA biosynthesis (Moss et al, 1983; Moss, 1985; McFadden and Dales, 1982) suggest that the virus might direct the biosynthesis of DNA precursors. The virus does not use products of host DNA breakdown (Parkhurst et al, 1972), and, therefore, viral DNA precursors are generated de novo. The supply of nucleotides may be limiting and lead to the cessation of viral DNA biosynthesis (McFadden and Dales, 1982). In studies with uninfected mammalian cells, several groups have confirmed the cytoplasmic location of host DNA precursor biosynthetic enzymes (Reddy and Pardee, 1980; Engström et al, 1984; Leeds et al, 1985).

Slabaugh et al. (1984) have reported that vaccinia virus infection results in virus-specific ribonucleotide reductase activity, which catalyzes the reduction of ribonucleoside diphosphates (NDPs) to deoxyribonucleoside diphosphates (dNDPs), the first reaction in the *de novo* pathway of DNA precursor biosynthesis. The virus encodes both a thymidine kinase (Hruby and Ball, 1982; Weir et al.,

1982), which is a salvage enzyme, and a DNA polymerase (Jones and Moss, 1984; Traktman et al., 1984). We have studied aspects of DNA synthesis in vaccinia virus-infected BSC-40 monkey kidney cells, specifically with respect to the action of antimetabolites and the interaction of DNA precursors and the DNA synthesis reaction. In order to characterize the role of virus-encoded enzymes of DNA precursor metabolism, we have isolated mutants resistant to inhibitors of DNA replication and studied the effects of these mutations on fidelity of DNA replication.

Study of action of antimetabolites in virus-infected cells and isolation of viral mutants resistant to the action of antimetabolites offer the opportunity of identifying virus-specific functions (Villarreal et al, 1984; Moss and Cooper, 1982; Sridhar and Condit, 1983; Raczynski and Condit, 1983) and of understanding the mechanism of drug action (Coen et al., 1982, Honess and Watson 1977, Hall et al., 1984). For example, the isolation of phosphonoacetic acid (PAA)- and arabinosyl adenine (araA)-resistant viral mutants has demonstrated that virus-specific DNA polymerases contribute to the toxicity of antivirals (Coen et al., 1982,; Moss and Cooper, 1982; Sridhar and Condit, 1983). This has facilitated the isolation and mapping of genes for viral enzymes (e.g., Jones and Moss, 1984; Traktman et al., 1984). Isolation of mutants resistant to inhibitors of DNA replication might clarify the role, if any, of nucleotides

in regulating DNA replication (Steinberg et al., 1979; Liu et al., 1983).

Drugs that inhibit mammalian DNA precursor metabolism have been useful in several respects. In the absence of well-defined mutants, drugs that are specific in their target offer a tool for manipulating metabolism in laboratory studies. In addition, drugs that affect DNA replication, an activity of growing cells or of viruses but not of terminally differentiated cells, have been useful in cancer and antiviral therapy. As discussed above, these drugs can also be used to isolate mutants in the metabolic pathways they affect. The nucleoside arabinosyl cytosine (araC), a 2'-hydroxy analogue of deoxycytidine, has been used in studies on nucleotide metabolism in mammalian cells (Robert de Saint Vincent et al., 1980; Robert de Saint Vincent and Buttin, 1980; Weinberg et al., 1981, 1985; Meuth et al., 1982). Mutants resistant to araC may be altered in enzymes of DNA precursor metabolism. The isolation of mutants resistant to araC and altered in replication fidelity (Meuth et al., 1979a, 1979b, 1982; Weinberg et al., 1981, 1985) confirmed the role of DNA precursor biosynthesis in maintaining fidelity of DNA replication. The relative accessibility of the viral genome as compared to the cellular genome and the relatively small number of viral genes as compared to the number of cellular genes makes a large virus such as vaccinia attractive for biochemical and

genetic studies. Studies using araC and vaccinia virus might clarify some aspects of the mechanism of araC toxicity as well. AraC is a useful antineoplastic agent (Clarkson et al., 1975). It is phosphorylated to 5' nucleotides by intracellular kinases. Ultimately, it damages DNA and causes cell death (Woodcock et al., 1979). AraCTP is the biologically active form of the drug. It is a competitive inhibitor of the incorporation of dCTP by DNA polymerase (Furth and Cohen, 1968; Momparler, 1969; Graham and Whitmore, 1970a, 1970b) causing DNA chain termination. Other mechanisms have been proposed, including the incorporation of araC into DNA causing subsequent chain termination (Kufe et al., 1980, 1984) and the inhibition of replicon initiation (Fridland, 1977a, 1977b; Bell and Fridland, 1980). The effects of other nucleosides or modulators of DNA precursor biosynthesis on araC toxicity have also been studied (e.g., Roberts et al., 1979; Grant et al., 1980; Ho et al., 1980; Walsh et al., 1980; Kinahan et al., 1981) for their relevance to the use of araC as a chemotherapeutic agent and as a probe of the complex interactions of DNA precursor biosynthesis.

Aphidicolin is a tetracyclic diterpenoid which interferes with replicative DNA polymerases, apparently by inhibiting incorporation of deoxynucleotides (Huberman, 1981; Frank et al., 1984; Defilippes, 1984). It has been demonstrated that certain drug-resistant herpesvirus DNA

polymerases are hypersensitive to aphidicolin (Coen et al., 1983; Honess et al., 1984). Furthermore, as aphidicolin is a competitive inhibitor of DNA polymerase α, alterations in nucleotide pools can contribute to aphidicolin resistance. In addition, certain aphidicolin-resistant mutants have shown altered replication fidelity (Liu et al., 1983; Hall et al., 1984). Some PAA-resistant herpesvirus mutants (which were more sensitive to aphidicolin than wild type) have shown increased replication fidelity compared to wild type (Hall et al., 1984); on the other hand a polymerase α mutant resistant to aphidicolin was hypermutable (Liu et al., 1983). However, Campione-Piccardo and Rawls (1984) have reported that mutants selected by PAA for altered polymerases show no change in mutability.

We have used the vaccinia virus system, with its potential for genetic and biochemical analysis, to ask whether there is a correlation between altered drug sensitivity and mutation frequency. We report here the isolation of vaccinia virus mutants resistant to araC. The isolation of viral mutants resistant to araC confirms that the virus encodes an enzyme or enzymes critical to the development of toxicity of this analogue of deoxycytidine and that virally encoded enzymes are involved in at least some steps of DNA precursor metabolism. AraC-resistant mutants that we have characterized are resistant to PAA but not to araA. Because some of the araC-resistant mutants we

studied were more sensitive than wild-type virus to aphidicolin, we have also examined some aphidicolin-resistant mutants for sensitivity to PAA and araC and measured relative mutation frequency. Among those mutants, we have found one that is hypersensitive to PAA and has a very high mutation rate, as determined by increased rate of occurrence of spontaneous resistance to the drug isatin ß-thiosemicarbazone (IBT) (Easterbrook, 1962; Woodson and Joklik, 1965; Bauer, 1972; Katz et al., 1973, 1978; Cooper et al., 1979; Rada et al., 1984) . To further understand the relationship between mutability and resistance to drugs that compete with DNA precursors (Hall et al.,1985; Liu et al., 1983), we have examined the effect of selecting derivatives of these mutants that are no longer hypersensitive. We report here the results of our investigations.

MATERIALS AND METHODS

Materials. Arabinosyl cytosine (araC), thymidine (TdR) and Phosphonoacetic acid (PAA) were from Calbiochemical. Aphidicolin was from the National Cancer Institute. Isatin ß-thiosemicarbazone (IBT) was a gift of R. Condit (SUNY-Buffalo).

Cell lines and virus strains. BSC-40 African green monkey kidney cells and vaccinia virus strain WR, the parent strain used in these experiments, have been described (Slabaugh et al., 1984). Cells were maintained in minimal essential medium (Irvine) containing 5% calf serum (Flow laboratories). Preparation of virus stocks was as described (Slabaugh et al., 1984). Plaques were visualized in 50% methanol/0.5% methylene blue after two days on monolayers. Crude stocks were dispersed by three cycles of freeze thaw and sonication (Condit and Motyczka, 1981); virus were allowed to adsorb for 15 minutes before addition of medium (containing or lacking drug as appropriate).

Measurement of mutation frequency. Plates of BSC-40 cells were inoculated with 25-50 plaque-forming units (pfu) and incubated until cytopathic effect was observed throughout the dish (Hall et al., 1984). Virus was harvested and frozen. Virus stocks were then dispersed and titrated in the absence of and in the presence of an inhibitory (greater than three orders of magnitude) concentration of IBT. For each experiment a wild-type control was used, as sensitivity

to a certain drug concentration were variable from experiment to experiment.

DNA Polymerase assay. DNA polymerase was assayed as previously described (Slabaugh et al.,1984).

RESULTS

Isolation of vaccinia virus mutants resistant to high concentrations of AraC

We have studied the effects of araC on vaccinia virus replication in mammalian cells. We would like to increase our understanding of deoxypyrimidine metabolism in this system as well as of the effects of alterations in pyrimidine metabolism on fidelity of DNA replication. The isolation of viral mutants resistant to araC, an analogue of deoxycytidine, would allow us to identify viral gene products involved in pyrimidine metabolism and allow the quantitation of effects of genetic alterations on replication accuracy.

As shown in Figure 2.1A, formation of plaques by vaccinia virus monolayers on BSC-40 cells is inhibited by araC. While the concentrations of drug that inhibit plaque formation are toxic to growing cells (data not shown), these amounts of drug do not interfere with the ability of confluent monolayers to act as host for viral plaque titration. After several days in medium containing levels of araC that completely inhibit plaque formation, the cells exclude trypan blue.

In order to select for spontaneous araC-resistant mutants, we passaged the virus in cells exposed to inhibitory concentrations of the drug. Cells were infected at various multiplicities (0.05-1.0 plaque forming

Figure 2.1

Plating efficiency of vaccinia virus on BSC-40 monolayers treated with arabinosyl cytosine (araC). Approximately 500 plaque forming units were added in 0.5 ml to 60-mm dishes. After a brief absorption period (about 30 minutes) inoculum was removed and the cells were overlaid with medium containing 1 % agar and the indicated concentration of drug. Cells were incubated for two days, stained with neutral red, and returned to 37º incubator (Condit and Motyczka, 1981). Averages are of two dishes. Error bars indicate standard deviation. (Absence of error bars indicates standard deviation =0.) A. Plaque formation in the presence of araC. B. Comparison of plaque forming ability in the presence (◆)or absence (⧈) of 4μM thymidine (TdR).

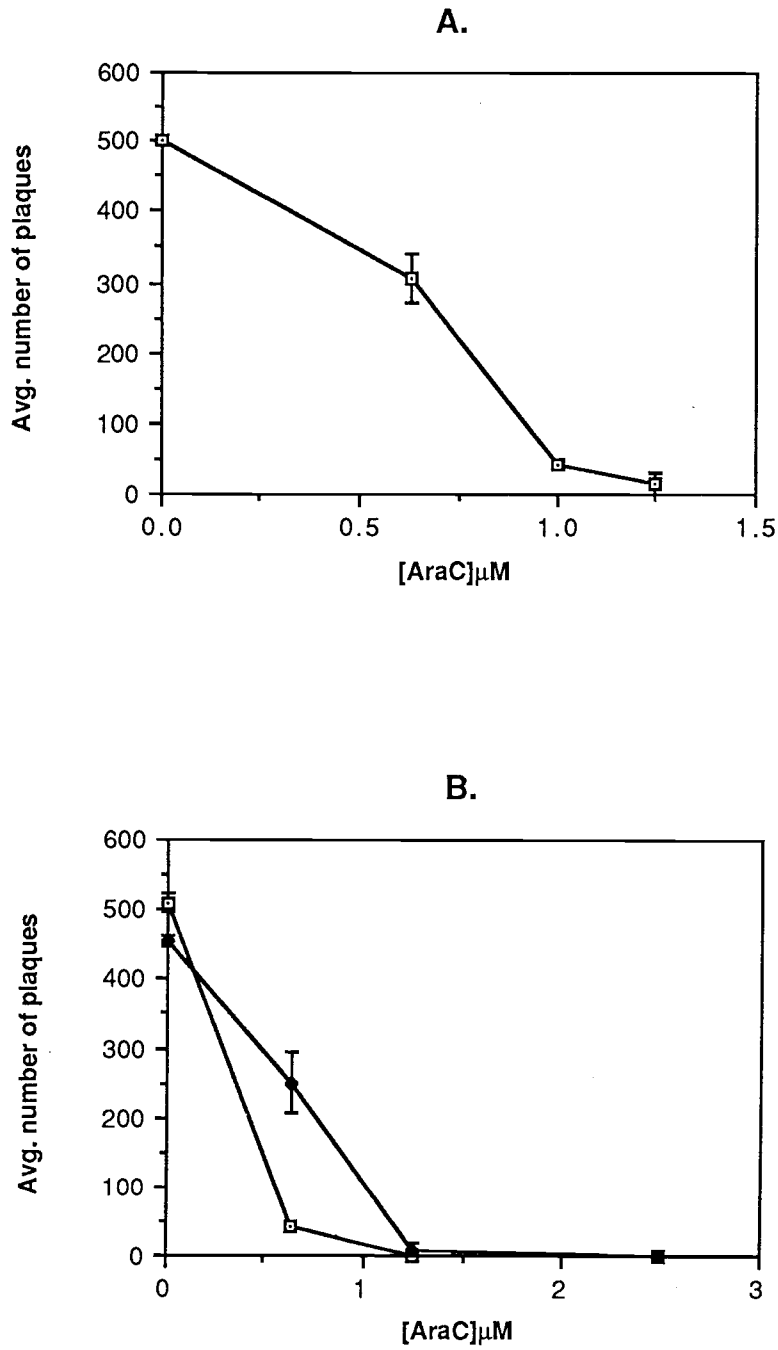
A.
Avg. number of plaques
600
500
400
300
200
100
0
0.0
0.5
1.0
1.5
[AraC]μM
B.
Avg. number of plaques
600
500
400
300
200
100
0
0
1
2
3
[AraC]μM

Figure 2.1

units/cell); virus was harvested after 2 days. The virus was titered on monolayers containing or lacking araC and the harvested virus was then used to infect monolayers which were maintained in the same concentration of drug as the earlier passage. Repeated attempts to select for a population of virus resistant to araC yielded no spontaneous mutants. Growth of virus passaged as many as seven times in araC was comparable to wild-type. In passaging the virus it is necessary to do parallel wild-type controls at each step, since the inhibitory concentration of drug is not consistent from experiment to experiment (data not shown).

We attempted to select mutants by increasing the concentration of araC from passage to passage, beginning with a concentration that was only slightly (about 20%) inhibitory to growth. As in the repeated passages in a more inhibitory concentration, this series yielded no virus population with increased resistance to araC (data not shown).

Certain araC-resistant mammalian cell mutants have altered sensitivity to thymidine and may require thymidine in order to grow (Meuth et al 1979). Figure 2.1B shows that the presence of a low concentration (4μM) of thymidine enhances wild-type virus plaque formation in the presence of araC. Pretreatment of cells with high concentrations of thymidine, 100μM, can increase araC cytotoxic effects (Grant et al., 1980) . Low level thymidine enhances the ability of

cells to support viral growth and plaque formation in the presence of araC. It is possible that the inability to select mutants is due to drug toxicity to the cells, such that the cells could not support virus growth. (This has been proposed as an explanation for the inability to isolate vaccinia virus mutants resistant to arabinosyl adenine. P. Saavedra and R. Condit, SUNY Buffalo unpublished).

Selection of mutants was therefore attempted in medium containing araC as well as thymidine. After the fourth passage stocks were enriched for virus able to grow in araC. Figure 2.2 shows the effect of selection on ability of the virus to grow in the presence of drug. Monolayers were infected at low multiplicity and harvested after there was extensive cytopathic effect. Crude extracts were assayed for virus titer. Virus was titered in the presence (Figure 2.2A) or absence (Figure 2.2B) of araC.

Individual plaques were isolated and stocks grown from these. Figure 2.3A shows the plaque-forming ability of one such isolate, designated $araC^r$-A. Figure 2.3B shows the plaque-forming ability of isolates from a separate selection for resistant virus as well as an isolate selected at the same time as $araC^r$A. After the selection of mutant virus, the isolated virus stocks could be grown in araC in the absence of thymidine (data not shown). The presence of thymidine was, therefore, necessary for the selection, but not for the growth, of the mutant viruses.

Figure 2.2

Effect of araC concentration on growth of (□) wild-type vaccinia virus and (◆) vaccinia virus passaged in the presence of 2.5μM ara C. Monolayers were infected with approximately 0.1 pfu/cell and harvested twenty-four hours later. Crude extracts were titrated on vaccinia vius monolayers A.) in the presence of 1.25μM araC and 2.5μM TdR or B.) in medium containing no added drugs. Log of titer is shown. The curve for wild-type virus indicates that the titer was less than 4000. Similar results were obtained in duplicate experiments.

Figure 2.3

Relative plating efficiency of wild-type and plaque-purified virus selected in araC. BSC-40 monolayers were inoculated with approximately 300 pfu/60 mm dish. After 15 minutes adsorption, viral inoculum was aspirated and replaced with fresh medium containing araC. After about 48 hours medium was removed and cells were fixed and stained with 50% methanol/0.5% methylene blue. A. Curve showing (□) wild-type and one (◆) mutant, $araC^r$-A. B. Other independently isolated mutants are compared with wild-type (□). Note differing sensitivity of wild-type in independent experiments. See text.

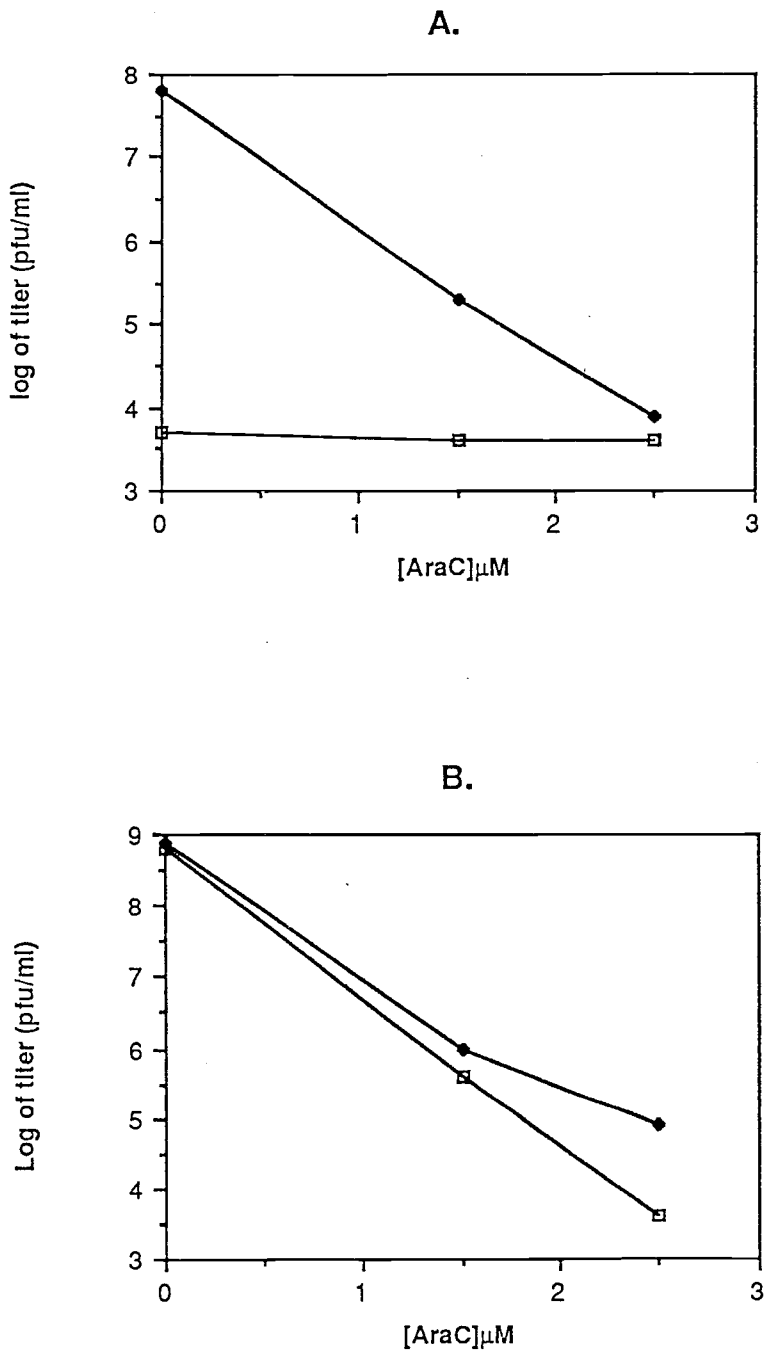
A.
log of tIter (pfu/ml)
8
7
6
5
4
3
0
1
2
3
[AraC]μM
B.
Log of tIter (pfu/ml)
9
8
7
6
5
4
3
0
1
2
3
[AraC]μM

Figure 2.2

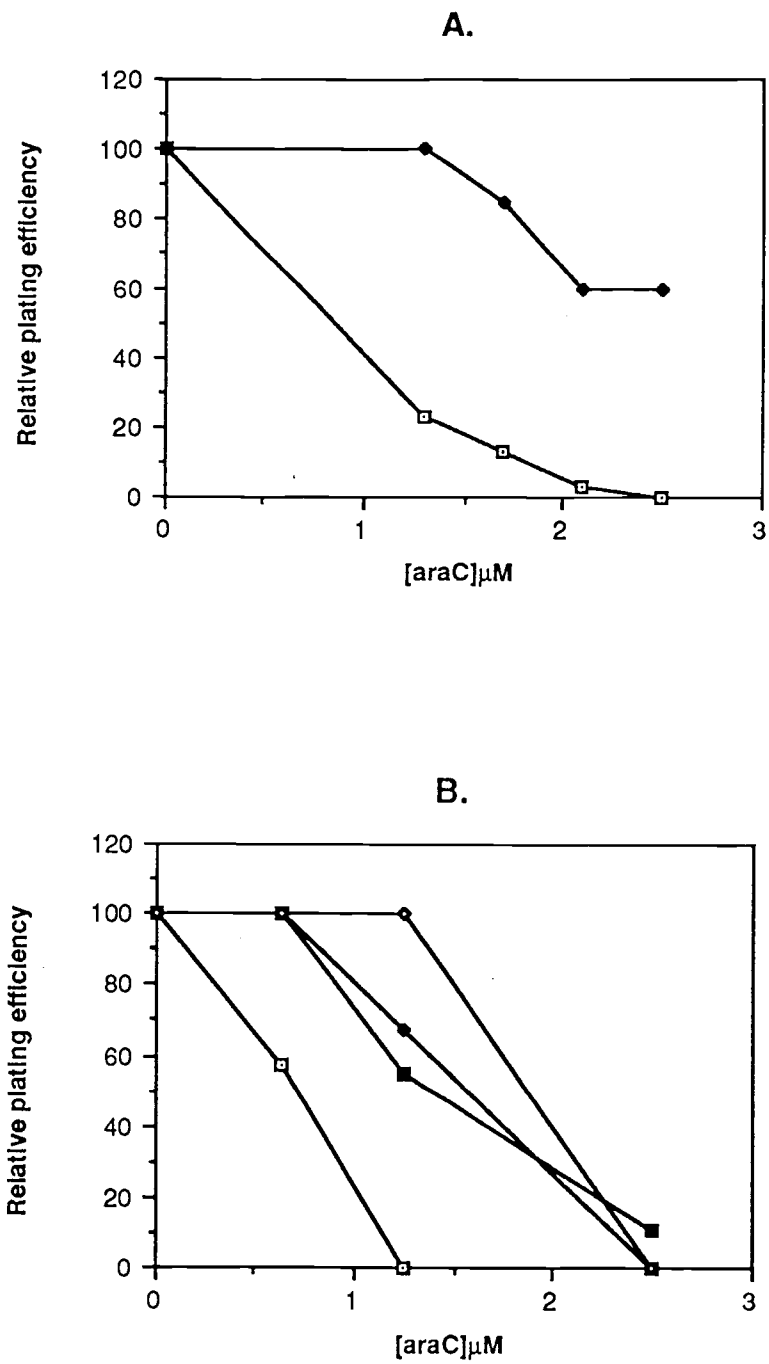
A.
Relative plating efficiency
120
100
80
60
40
20
0
0
1
2
3
[araC]μM
B.
Relative plating efficiency
120
100
80
60
40
20
0
0
1
2
3
[araC]μM

Figure 2.3

Partial characterization of the araC-resistant mutants

AraC must be phosphorylated, first to the nucleoside monophosphate and ultimately to the nucleoside triphosphate, which is the inhibitor of the DNA polymerase (Cohen 1966). Resistance to the drug can be due to the inability of one of the kinases to phosphorylate the arabinose analogue or to the discrimination by the DNA polymerase (Meuth et al., 1979; Kufe et al., 1984). In addition, metabolic alterations that cause an increase in the level of deoxycytidine nucleotides allow for successful competition between the normal nucleotide and the analogue (Meuth et al., 1979; Weinberg et al., 1981). Alterations that cause an increase in the level of deoxycytidine nucleotides mitigate the effectiveness of the analogue by competition between the normal and abnormal nucleotides.

In order to evaluate the mutants that we have isolated we measured the activity of DNA polymerase in extracts from cells infected with wild-type or mutant virus. We did this in the presence of various concentrations of ara-CTP. In addition, we studied the resistance of the mutants to other DNA polymerase inhibitors, aphidicolin (Huberman, 1981) and phosphonoacetic acid (PAA) (Honess and Watson, 1977). Mutations in herpes simplex virus that give rise to PAA resistance have been mapped to DNA polymerase (Jones and Moss, 1984; Traktman et al., 1984; Coen et al., 1984) . Such mutants often show altered sensitivity to other drugs

that inhibit DNA polymerase (Cohen et al., 1983; Honess et al., 1984).

Figure 2.4 shows the result of an experiment comparing the DNA polymerase activity in crude extracts from mock-, wild-type-, or mutant-infected cells. The extracts from the mutant-infected cells are completely resistant to the effects of 60μM araCTP, a concentration which partially inhibits the activity in wild-type-infected cells as well as in mock-infected cells.

Figure 2.5A shows the results of plaque titration in the presence of phosphonoacetic acid. Some, but not all, of the isolates are resistant to PAA. Figure 2.5B shows that some of the araC-resistant mutants are more sensitive to aphidicolin than is wild-type virus. This is consistent with observations made in herpesvirus with araA-, PAA-, and aphidicolin-resistant mutants (Coen et al., 1983; Honess et al., 1984). However, it should be noted that none of the araC-resistant mutants that we tested showed altered sensitivity to araA (data not shown). Herpesvirus mutants resistant to araA can be resistant to araC as well (Kufe et al., 1984). Saavedra and Condit (unpublished) were unable to isolate vaccinia virus mutants resistant to araA.

Among mutants selected for ability to form plaques inPAA, some are araC-resistant (Figure 2.6). Since such PAA^r-$AraC^r$ mutants can be isolated after one passage in 90%-inhibitory concentrations of PAA (data not shown),

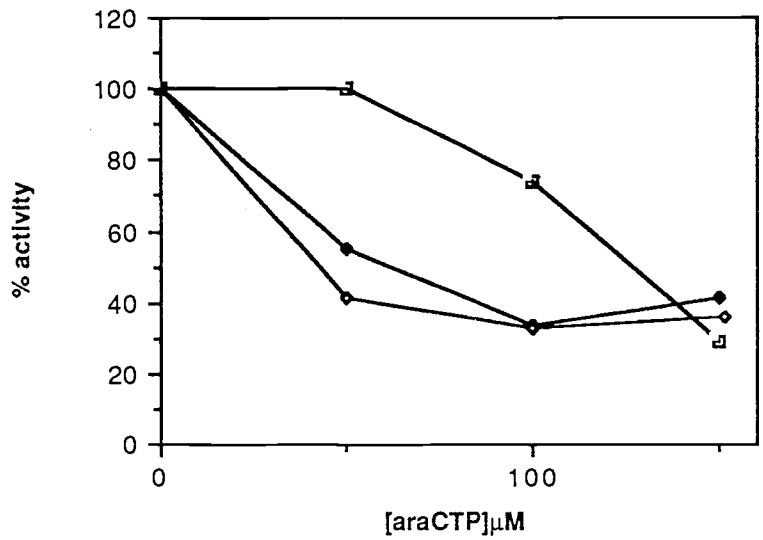

Figure 2.4. Relative DNA polymerase activity in the presence of various concentrations of the inhibitory nucleoside triphosphate araCTP. (◆) wild-type virus; (◇) mock-infected cells; (◻) araC -3 virus.

Figure 2.5

Relative plating efficiency of araC^r mutants of vaccinia virus in the presence of other drugs. Approximately 30-50 pfu (per well of a 24-well dish) were adsorbed to BSC-40 monolayers for 15 minutes. Inoculum was aspirated and replaced with medium containing the indicated drug. Cells were stained with methylene blue and plaques counted. Similar results were obtained in duplicate experiments. A.) In the presence of phosphonoacetic acid (PAA): (⧈) wt, araC^r-3, araC^r-9; (◆) araC^r-4. B.) In the presence of aphidicolin: (⧈) wt; (◆) araC^r-2, araC^r-3, araC^r-Z; (■) araC^r-9.

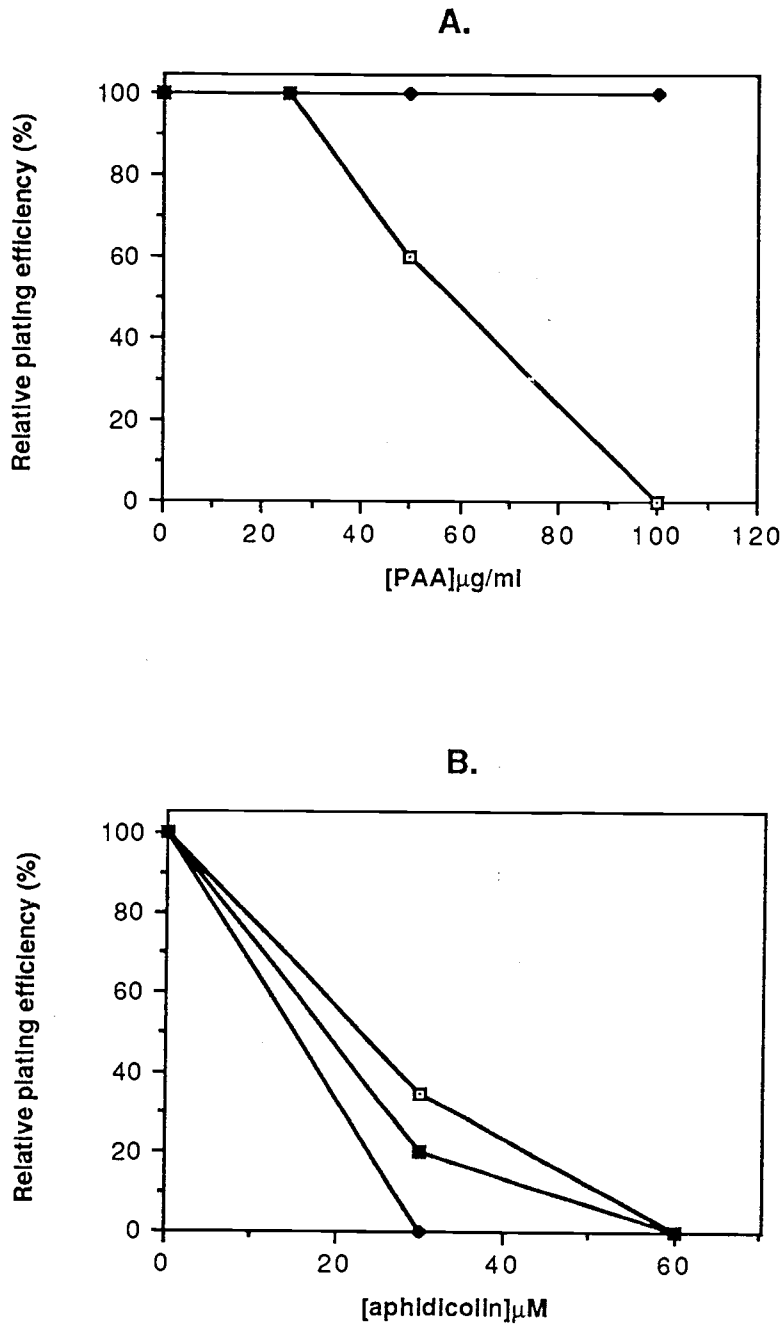
A.
Relative plating efficiency (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
[PAA]µg/ml
B.
Relative plating efficiency (%)
100
80
60
40
20
0
0
20
40
60
[aphidicolin]µM

Figure 2.5

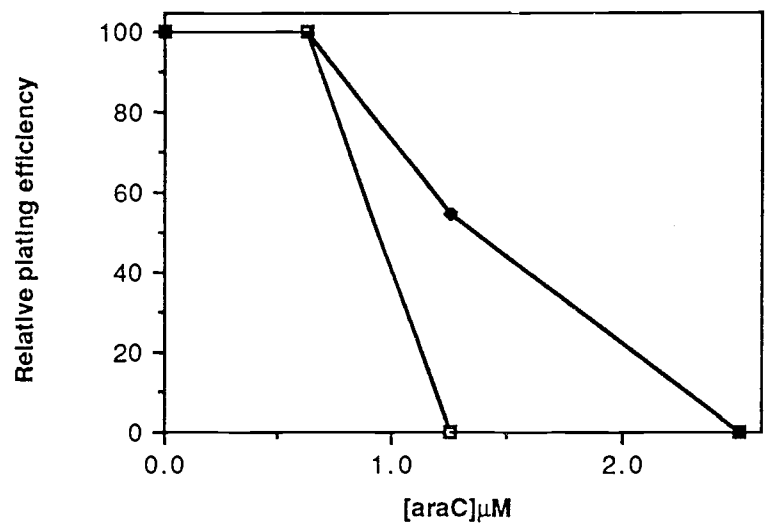

Figure 2.6. Relative plating efficiencies of phosphonoacetic acid (PAA)-selected mutants in the presence of arabinosyl cytosine (araC). (▫) wild-type and PAA^r-4; (◆) PAA^r-1.

resistance to araC occurs often. Selection in araC, however, was difficult. As a selective agent for isolating DNA polymerase mutants, araC is less effective than PAA, even though some of the mutants have similar properties according to our preliminary characterization.

These results show that the mutants isolated as resistant to araC have varied properties with respect to degree of sensitivitiy to araC, aphidicolin and PAA. That is, they are not all derived from a single mutant virus. All are resistant to araC and hypersensitive to aphidicolin. Some are resistant to PAA. DNA polymerase assays in crude extracts demonstrate that the mutants are altered in that enzyme, which would account for all the above observations.

Mutation frequencies of araC-resistant mutants

In order to determine relative mutation frequencies, BSC-40 monolayers were infected with a small number of plaque-forming units and virus-infected cells were harvested after cytopathic effect was observed throughout (Hall et al.,1984). Virus was titered in the presence and absence of istatin ß-thiosemicarbazone (IBT) (Easterbrook, 1962; Bauer, 1972; Katz et al., 1973, 1978; Cooper et al., 1979; Rada et al., 1984) to determine the fraction of IBT-resistant virus. Table 2.1 shows the relative mutation frequencies.

Hall et al. (1984) have demonstrated that some herpesvirus DNA polymerase mutants have a lower mutation

Table 2.1

Mutant Fraction of Viral Isolates

Viral Isolate	Mutant fraction	Relative frequency*
EXPERIMENT 1		
Wild-type	5.5×10^{-4}	1.0
$AraC^r$-1	4.0×10^{-4}	0.7
-2	5.0×10^{-5}	0.9
-3	3.0×10^{-5}	0.05
-9	9.0×10^{-5}	0.2
-Z	2.5×10^{-4}	0.5
EXPERIMENT 2**		
Wild-type	1.0×10^{-4}	1.0
PAA^r3-1	2.0×10^{-4}	2.0
-2	1.0×10^{-4}	1.0
-3	2.0×10^{-4}	2.0
-4	1.0×10^{-4}	1.0
-5	7.0×10^{-4}	7.0

*The frequency of mutations in wild-type is considered to be 1.0. Note the difference in fraction of wild-type virus resistant to IBT in independent experiments.
**In experiment 2, the mutants are individual isolates from viral stocks that have been through three rounds of growth on monolayers fed with medium containing phosphonoacetic acid.

frequency than wild-type. This was true for mutantsselected for resistance to PAA or araA. Among those were mutants that were hypersensitive to aphidicolin. The results in Table 2.1 show that some of the vaccinia virus mutants selected for resistance to araC are somewhat less mutable than the wild-type. Among vaccinia virus mutants selected for resistance to PAA and examined for relative mutation frequencies there are none with lower mutation frequencies than the wild-type virus and one with a seven-fold higher frequency (Table 2.1).

Liu et al. (1983) have isolated a cellular mutant resistant to aphidicolin and with a mutator phenotype. We examined the possibility that resistance to aphicicolin in viruses might similarly correlate with mutation frequency. We have measured the relative mutation frequency of vaccinia virus mutants selected by M. Slabaugh for resistance to aphidicolin. Among these, one mutant, aph^r C-10, was resistant to aphidicolin but hypersensitive to araC as well as PAA.

Aph^r C-10 had a very high mutation frequency: typically the fraction of IBT-resistant/total pfu was about twenty times that of wild-type (Table 2.2). In addition we estimated that in the population of aph^r C-10 nearly one per cent of the plaques were no longer hypersensitive to PAA. Since this mutation is at a different locus, the reversion frequency confirms the mutability of the virus. Because

Table 2.2

Mutant fraction of viral isolates

Viral isolate	Mutant fraction	Relative frequency
Wild-type	6×10^{-4}	1.0
AraC^{r}A	1×10^{-4}	0.2
	5×10^{-4}	0.8
	2×10^{-4}	0.3
Aph^{r}C-10*	1×10^{-2}	17
	4×10^{-2}	67
	1×10^{-2}	17
	1×10^{-3}	2
	7×10^{-2}	117
	1×10^{-2}	17

			Drug resistance		
			aph	PAA	araC
Wild-type	9×10^{-5}	1.0	0	0	0
Aph^{r}C-10	2×10^{-3}	20	++	--	--
R2-1**	6×10^{-5}	0.7	N.D.	N.D.	N.D.
R2-2	3×10^{-4}	3.3	++	+	-
R2-3	2×10^{-5}	0.2	++	+	0
R2-4	2×10^{-3}	20	+	+	0
R2-5	1×10^{-4}	1.1	N.D.	N.D.	N.D.
R2-6	1×10^{-4}	1.1	N.D.	N.D.	N.D.

+=resistant
0=wild-type
-=sensitive

*Six individual preparations grown from one or a few plaque-forming units of the mutant were analyzed for IBT resistance.
**Mutants designated R2-1 through R2-6 were grown twice in concentrations of phoshonoacetic acid (PAA) which were not inhibitory to wild-type but which were inhibitory to the parent aph^{r}C-10 (see text). These mutants have, therefore, "reverted" from hypersensitivity to PAA.

mutants arose so frequently (as many as one IBT-resistant plaque-forming unit per one hundred pfus), we altered our procedure for determining relative mutation frequency. Rather than infecting monolayers with 25-50 pfu/60 mm dish, we have diluted the virus to infect with about one pfu per dish. Table 2.2 shows the results of one such experiment comparing the relative mutation frequencies of $araC^r$ A, wild-type, and aph^r C-10. Of the six crude preparations titered for IBT resistance, five showed high relative mutation rates. In contrast, the araC-resistant mutant is somewhat less mutable than wild-type. With respect to rate of growth and titer the C-10 mutant is comparable to wild-type (data not shown).

Altered senstitivity to inhibitors and mutation frequency

We exposed aph^r C-10 to 30μg/ml PAA, a concentration that does not inhibit wild-type virus but which does inhibit C-10. We examined the "revertants" to wild-type resistance to PAA for mutability as well as drug resistance. Table 2.2 shows the results of relative mutation frequency studies on the "revertants" as well as the level of resistance of virus isolates to three drugs: araC, aphidicolin and PAA.

Since C-10 showed very high relative mutation frequency and altered sensitivity to three drugs, we examined the effect of an alteration in one of the properties of C-10, that is, the sensitivity to PAA, on the others. Of the six

we examined, one was hypermutable. This virus, called C-10r2-3, was somewhat less resistant to aphidicolin than C-10. It was more resistant than wild-type to PAA and about like wild-type in sensitivity to araC. Thus, its drug resistance pattern was quite different from that of C-10 even while it remained hypermutable.

The other "revertants" had mutation frequencies closer to that of wild-type. Two mutants examined for resistance to aphidicolin remained as resistant as C-10; C-10r2-3, however, had a mutation frequency somewhat lower than wild-type.

DISCUSSION

We have isolated viral mutants resistant to the nucleoside ara-C. Ara-C is an analogue of deoxycytidine which is phosphorylated to ara-CTP. Ara-CTP is an inhibitor of DNA biosynthesis which acts by inhibition of DNA polymerase (Furth and Cohen, 1968; Momparler, 1969; Graham and Whitmore, 1970a, 1970b), by incorporation of the ara-C into DNA (Kufe et al., 1980, 1984), or by inhibition of replicon initiation (Fridland, 1977a, 1977b; Bell and Fridland, 1980). Since the drug is useful in cancer chemotherapy (Clarkson et al., 1975), the mechanism of action is of interest. We have examined the effects of the drug on vaccinia virus replication to understand the coordination of DNA precursor metabolism and fidelity of DNA replication. We have also studied certain aphidicolin-resistant viral mutants with respect to fidelity of DNA replication.

Both ara-CTP (Furth and Cohen, 1968; Momparler, 1969; Graham and Whitmore, 1970a, 1970b) and aphidicolin (Frank et al 1984) have been considered competitors with dCTP and, we have, therefore, isolated mutants that are potentially altered with respect to the metabolism of dCTP (Liu et al., 1983). In our preliminary characterization, we have examined the nature of the mutants and the effects of the mutations on resistance to other drugs and on mutation frequency. Multiple drug resistance might be accounted for

by mechanisms similar to those proposed for herpesvirus DNA polymerase drug-resistant mutants--alterations in a domain that codes for dNTP and pyrophosphate binding sites (Gibbs et al.,1985; Hall et al.,1985).

The frequency of the mutations suggests that they are due to single events. However, the alterations in patterns of resistance are not predictable. For example, while hypersensitivity to aphidicolin correlated with resistance to PAA or to araC, the reversion to a wild type sensitivity to PAA or araC was not correlated with a return to aphidicolin sensitivity or to normal mutability (Honess et al., 1984). Recent reports suggest that the altered mutation rate observed in HSV mutants might be due to the selection of nucleotides (Hall et al 1984, Hall et al 1985); the binding sites for dNTP analogues, aphidicolin, and PAA map to the C-terminus (Gibbs et al 1985) and such localization of binding sites might account for multiple drug resistance (Gibbs et al 1985, Coen et al 1983, Honess et al 1984). The HSV polymerase differs from that of vaccinia virus. Both have 3' exonucleolytic editing functions, but the polymerases differ somewhat in sensitivity to inhibitors (Kornberg 1982). The vaccinia polymerase is somewhat less sensitive to aphidicolin than is the cellular α polymerase (Defillipes 1984). We have studied the nature of resistance to aphidicolin and possible correlation with mutability. Resistance to aphidicolin

might result from a less fastidious polymerase that is therefore more likely to incorporate an erroneous nucleotide but less likely to bind the inhibitor (Liu et al.,1983; Hall et al.,1985; Gibbs et al.,1985); conversely the aphidicolin-hypersensitive, araC-PAA-resistant mutants might be more selective in binding nucleotides, allowing greater aphidicolin binding (and lower mutation rates due to improved nucleotide selection) (Hall et al.,1984; 1985).

Our PAA- and ara-C-resistant mutants do not show a drastic reduction in mutation rates, although they do show altered sensitivity to several drugs. To pursue the possible relationship between aphidicolin sensitivity and mutability we studied an aphidicolin-resistant mutant with a mutation frequency about twenty times higher than wild-type. As suggested above, we considered that aphidicolin resistance correlates with higher mutation rates because a rapid turnover of dNTP substrate reduces the inhibitory binding of aphidicolin (Hall et al.,1985). The polymerase is, therefore, less selective. However, among spontaneous mutants derived from a viral mutant with abnormally high mutation rates and resistance to aphidicolin, there was an isolate with altered sensitivity but no change in mutation rate and others which were as resistant to aphidicolin but no longer had a higher than wild-type muation frequency.

Among the mutants we have studied we found none resistant to the antiviral araA (North, 1984), in contrast

with observations in the herpesvirus system (Coen et al.,1983, Kufe et al.,1984). P. Saavedra and R. Condit (SUNY, Buffalo, unpublished) were unable to select vaccinia virus mutants in araA. Our own inability to select araC-resistant mutants in medium containing araC (without added thymidine) emphasizes the importance of the selection system. However, the lack of araA-resistant mutants among viruses selected either for araC- or PAA-resistance is somewhat surprising given the frequent multiple alterations in drug resistance that we and others have observed (Coen et al., 1983; Honess et al., 1984) and the suggestion that such mutants have an altered ability to select nucleotides (Hall et al.,1985). The altered sensitivity to araC presumably involves altered recognition of the sugar moiety. However, the alteration does not extend to altered selection of araATP.

Furthermore, selection in araC resulted in mutants of differing sensitivities to drug and differing mutation frequencies. The mutants that we have characterized are DNA polymerase mutants: they show altered sensitivity to other DNA polymerase inhibitors as well as to araC. This does not eliminate the possibility that some component of the resistance to araC arises from alterations in the enzymes responsible for synthesizing the dNTPs. The virally induced ribonucleotide reductase (Slabaugh et al.,1984; Slabaugh and Mathews, 1984) would be one enzyme whose alteration might

lead to decreased sensitivity to araC (Meuth et al., 1979b). While the isolation of drug-resistant viral mutants confirms that the virus encodes the gene product, the inability to isolate mutants does not mean that several enzymes of nucleotide metabolism are not virally encoded. Our own difficulty isolating ara-C-resistant mutants suggests that the selection scheme is critical.

Chapter III. STIMULATION OF DNA SYNTHESIS IN CONFLUENT BSC-40 MONOLAYERS INHIBITS VACCINIA VIRUS PLAQUE FORMATION AND SENSITIZES VIRUS SPECIFICALLY TO METHOTREXATE

INTRODUCTION

Because the synthesis of deoxyribonucleoside triphosphates (dNTP) is closely linked with DNA replication, the study of the biosynthesis of these DNA precursors in eukaryotic systems should increase understanding of fidelity of DNA replication, genetic consequences of nucleotide pool imbalances, temporal control of DNA replication, and the actions of antimetabolites (Mathews and Slabaugh, 1986). We have studied some aspects of DNA precursor biosynthesis during infection of mammalian cells by vaccinia virus, a large, double-stranded DNA-containing virus.

Vaccinia virus replicates its genome in the cytoplasm (Moss 1985), away from the site of cellular DNA replication. In addition the large genome (about 180 kb) has coding capacity for proteins that have not been identified (Moss et al., 1983, McFadden and Dales, 1982; Moss, 1985). About one half of the viral genome is transcribed prior to DNA replication (Kaverin et al., 1975), and there are about one hundred early genes distributed throughout the vaccinia genome (Belle Isle et al., 1981). Some have been localized but only a few identified (Moss, 1985). The relative accessibility of viral genomes,as compared to cellular

genomes, makes a large DNA virus like vaccinia attractive for combined genetic and biochemical investigations of DNA precursor metabolism in mammalian cells.

We have used biochemical and genetic approaches to understand the relationship between cellular and viral dNTP biosynthesis and to relate dNTP biosynthesis to mutation frequencies and the action of certain antimetabolites. Slabaugh et al. (1984) have reported that vaccinia virus induces ribonucleotide reductase, the enzyme that catalyzes the first unique step in the synthesis of DNA; ribonucleotide reductase catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates (Thelander and Reichard, 1979). Recent studies (M. Slabaugh and R. Davis, unpublished) confirm that this enzyme is virus-coded.

Another enzyme specific to the biosynthesis of deoxyribonucleotides is thymidylate synthase, which catalyzes the conversion of deoxyuridylate to thymidylate in a reaction that oxidizes 5,10-methylene tetrahydrofolate to dihydrofolate (Figure 3.1). Thymine nucleotides normally occur only as deoxynucleotides. For this reason the reaction catalyzed by thymidylate synthase has been a target for cancer chemotherapy, which is aimed at proliferating cells (Danenberg, 1977). A reduction in the level of thymidylate biosynthesis after depletion of reduced folates has caused some to suggest that inhibition of thymidylate

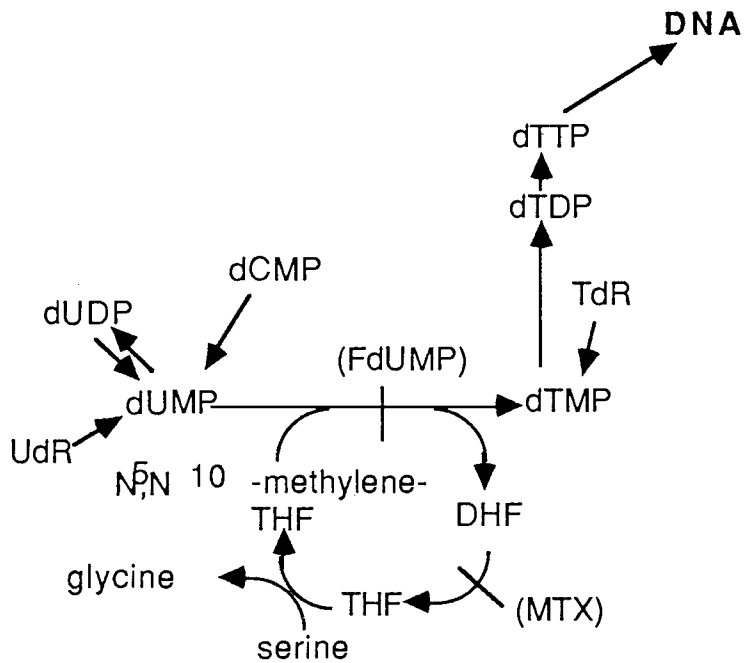

Figure 3.1. Biosynthesis of thymidylate (dTMP) and reduction of dihydorofolate (DHF) to tetrahydrofolate (THF). Abbreviations are DNA, deoxyribonucleic acid; FdUMP, fluorodeoxyuridylate; MTX, methotrexate; dUMP, deoxyuridylate; dUDP, deoxyuridine diphosphate; UdR, uridine; dCMP, deoxycytidylate; TdR, thymidine; dTDP, thymidine diphosphate; dTTP, thymidine triphosphate.

biosynthesis leads to a "thymineless death" analogous to that described in bacterial mutants auxotrophic for thymidine (Cohen and Barner, 1954; Rueckert and Mueller, 1960; Cohen and Studzinski, 1968; Borsa and Whitmore, 1969; Danenberg, 1977; Goulian et al., 1980b; Ingraham et al., 1982; see below).

As mentioned above tetrahydrofolate is oxidized during the biosynthesis of dTMP (Figure 3.1). Since reduced folates are required for the biosynthesis of purines, glycine, and methionine as well as of thymidylate, the reduction of folates after oxidation in thymidylate biosynthesis is critical. Inhibition of dihydrofolate reductase by the folate analogue methotrexate (MTX), coupled with the oxidation of folates by thymidylate synthase, renders cells unable to replenish the pool of tetrahydrofolate, leading to cell death (Moran et al, 1979). These points have been exploited in chemotherapy as well as in experimental systems: thymidylate synthase (Danenberg, 1977) and dihydrofolate reductase (Kisliuk and Brown, 1977) have been specific targets for chemotherapeutic attack. We have exploited the properties of MTX and of 5-fluorodeoxyuridine (FUdR), which when phosphorylated to 5-fluorodeoxyuridylate is a virtually irreversible inhibitor of thymidylate synthase (Danenberg, 1977), to explore aspects of deoxyribonucleotide metabolism in vaccinia-infected cells.

Drug resistance has been exploited as a means of identifying virus-specific functions (Villarreal et al 1984,Moss and Cooper 1982,Sridhar and Condit 1983,Raczynski and Condit 1983). We have attempted the isolation of viral mutants with altered sensitivity to the nucleoside analogue FUdR and to the folate analogue MTX. Several mechanisms for resistance to MTX have been reported in whole animals and in cultured mammalian cells (Schimke, 1984; Schimke et al., 1985). We have also attempted to isolate viral mutants resistant to high levels of FUdR, perhaps via altered affinity of thymidylate synthase for the FdUMP or by altered pools of dUMP, the substrate for the enzyme (Jackson, 1978). We have not, by the selection procedures described in this report, isolated viral mutants with altered sensitivity to either of these two anti-metabolites. The isolation of viral mutants resistant to the drug would be strong evidence for a virally encoded enzyme. The negative result is less conclusive. However, particularly with MTX selection, the lack of mutants suggests that the virus does not encode a dihydrofolate reductase.

However, during our investigations we noted a specific effect of host cell DNA synthesis on MTX inhibition of viral replication. We have observed that stimulation of host cell DNA replication and, concomitantly, of host cell dTMP biosynthesis renders viral replication particularly sensitive to methotrexate, suggesting that the burden on the

host cell dihydrofolate reductase is increased by viral replication. We have observed that FUdR is also an inhibitor of viral replication. Assays of thymidylate synthase (in situ) and of dihydrofolate reductase (in vitro) show no stimulation of enzyme activity after viral as compared to mock infection.

Vaccinia virus relies on *de novo* biosynthesis of DNA precursors (Parkhurst et al., 1972). The extreme sensitivity to MTX after stimulating host cell DNA synthesis and the lack of genetic or biochemical evidence for vaccinia encoded thymidylate synthase or dihydrofolate reductase suggest that for certain enzymes of *de novo* DNA precursor metabolism the virus must rely on host activities. On the other hand, for at least one critical activity, ribonucleotide reductase (Slabaugh et al 1984) the virus induces its own enzyme.

MATERIALS AND METHODS

Drugs. Methotrexate (MTX), fluorodeoxyuridine (FUdR), and phosphonoacetic acid (PAA) were from Calbiochem. Isatin ß-thiosemicarbazone (IBT) was a gift of R. Condit (SUNY-Buffalo). ^{3}H-Thymidine was from ICN Radiochemicals.

Cells and virus. BSC-40 cells and vaccinia virus have been described previously (Slabaugh et al., 1984; this thesis, chapter 2). For determination of effects of pre-treatment on subsequent growth and plating efficiency of virus, the following procedure was used. BSC-40 cells were plated and allowed to reach confluency (about 48 hours). At the time indicated for pre-treatment, medium was aspirated and fresh growth medium (with or without added drug,as indicated) was added to the cells. At the time indicated for infection (time=0), medium was aspirated and growth medium containing virus inoculum was added to the monolayers. Virus was allowed to adsorb for 15-20 minutes. Inoculum was then aspirated and medium with indicated amount of drug was added to the cell monolayers. Cells were stained with methylene blue/methanol at 40-48 hours post-infection and plaques counted. Where so indicated, medium change at time=0 means that there was a change of medium immediately prior to infection.

Autoradiography. To visualize nuclei incorporating radioactive DNA precursors, the procedure of Izant and Weintraub (1984) was used. Nuclei were counted in a light

microscope (Olympus). Those nuclei with greater than eight grains were positive. Twenty fields per dish were counted.

Enzyme assays. Thymidylate synthase activity was determined *in situ* as described by Rode et al.(1980). Dihydrofolate reductase activity in cell extracts was measured spectrophotometrically (Mathews and Huennekens, 1963). Experiments were done in parallel, but the activity of thymidylate synthase was measured by labeling living cells.

RESULTS

Plaque reduction in presence of increasing [MTX] following various medium changes

In order to characterize pyrimidine metabolism in vaccinia virus-infected cells, we have examined the effects of various metabolic inhibitors on viral growth and on the ability of virus to form plaques. We have compared, as well, the effects of pre-treatment, i.e. incubation of monolayers in the presence of various inhibitors for varying periods prior to infection. By treating cells with inhibitors prior to infection, cellular targets might be preferentially inhibited thus amplifying the signal of virally induced enzymes after infection.

Methotrexate (MTX), a folate analogue, inhibits the enzyme dihydrofolate reductase (Nichol and Welch, 1950; Osborn et al., 1958; Kisliuk and Brown, 1977), which reduces folates oxidized in the synthesis of thymidylate from deoxyuridylate, a critical reaction in the biosynthesis of DNA precursors (Danenberg, 1977; Moran et al, 1979).

Fig. 3.2 shows the effects of a 9 hour incubation, prior to infection, of BSC-40 monolayers in high concentrations of MTX. Incubation in the presence of 20 or 50 μM MTX did, indeed, reduce the ability of the virus to form plaques as compared to virus titrated on cells not previously exposed to drug. After infection, the result of the pretreatment was most apparent in concentrations of MTX

up to 2 μM.

There was a difference between two sets that had not been exposed to MTX before the viral infection. The medium from one set of dishes was aspirated and replaced with fresh growth medium containing no drug ("0μM MTX") at the same time as fresh medium containing 20μM or 50μM MTX was added to other series of dishes. One set of dishes was not treated; that is, the cells were maintained in the medium that had been on the dishes since the cells were plated ("No Change"). Furthermore, the virus-infected BSC-40 monolayers that were not treated with MTX but had been given fresh medium (without drug) 9 hours before infection, had fewer plaques than the control (no MTX) monolayers that had no medium change from the time the cells were plated until virus infection.

The protocol for these experiments was as follows. Cells to be used as host for plaque titration were grown to confluence (usually 48 hrs after plating). When the monolayers reached confluence (Time=0 hours), one set of monolayers was given fresh growth medium while the medium on a parallel set of dishes was left unchanged. At Time=9 hours, approximately 550 plaque forming units were added to each dish. For those dishes that had a medium change at Time=0, growth medium was aspirated; virus was added in a small amount of medium and allowed to adsorb for 15-20 minutes, at which time the inoculum was aspirated and drug-

containing medium (or control medium lacking drug) was added to the cells. For those cells that had not received fresh medium at time 0 ("No Change") the protocol at time of infection was altered slightly:. Medium was aspirated, fresh medium was added to the dish, and then that medium was aspirated and viral inoculum added to the dish, from which point the two sets of monolayers were treated identically. In other words these monolayers also had a medium change, but there was no time lag between that medium change and the subsequent infection.

Two effects of adding fresh medium *without MTX* several hours before infection are apparent from Figure 3.2. First, the monolayers that received fresh medium without MTX several hours before the plaque titration had fewer plaques than monolayers that did not receive fresh medium from the time of plating of the BSC-40 cells until immediately before infection (Figure 3.2A). The fresh medium led to a reduced number of plaques. Second, the slopes of the curves for the two sets of dishes that received medium without MTX (one at 9 hours before infection and one at 0 hours before infection) differ. Medium change several hours before infection increased the sensitivity of the virus to MTX.

Furthermore, in this experiment, the pretreatment with MTX seemed to have the greatest effect (compared with cells given fresh medium without drug) on the subsequent plaque reduction in the presence of MTX up to 2μM. The curves of

Figure 3.2

Effect of pre-treatment of BSC-40 monolayers on subsequent plaque formation by vaccinia virus in the presence of methotrexate. Nine hours before viral infection confluent monolayers were left untreated (□) or were given fresh medium containing 0 μM (◆), 20 μM (■), or 50 μM (◇) MTX. At the time of infection, medium was aspirated and about 550 pfu/ 60-mm dish were added in 0.5 ml of growth medium. After 15 minutes adsorption, inoculum was aspirated and medium containing MTX at 0, 2, 10, 20, or 50 μM was added. After about 2 days, plaques were visualized by staining cells with methanol/methylene blue. A. Average number of plaques from two dishes. Error bars show standard deviation (absence of error bars indicates standard deviation =0). B. Plating efficiency as a percentage of the number of plaques at 0 μM MTX for each pre-treatment.

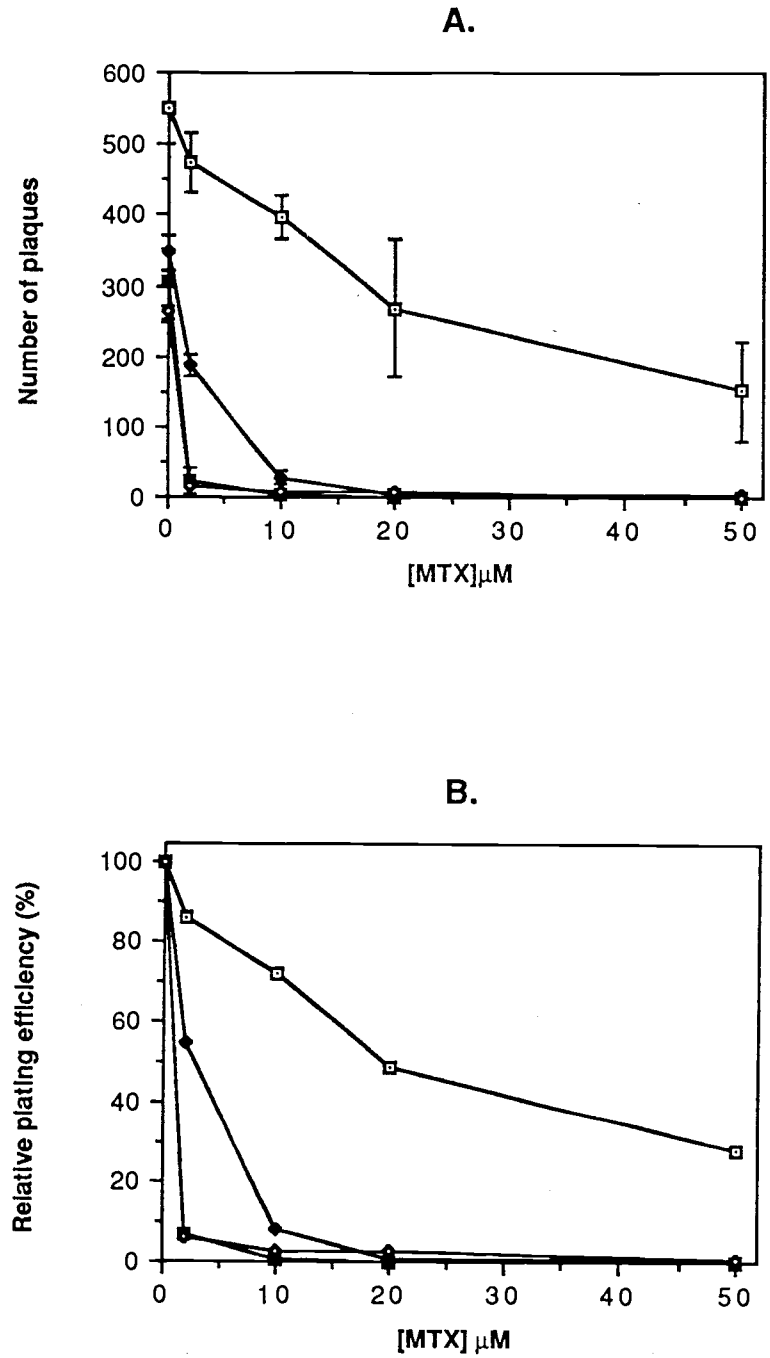
A.
Number of plaques
600
500
400
300
200
100
0
0
10
20
30
40
50
[MTX]μM
B.
Relative plating efficiency (%)
100
80
60
40
20
0
0
10
20
30
40
50
[MTX] μM

Figure 3.2

the three sets given fresh medium (with or without drug) were markedly different from the set which had no medium change prior to the plaque assay. Figure 3.2B shows the percentage of relative plating efficiency. That is, for each pretreatment, the number of plaques which form in the absence of MTX is considered 100%.

Very high MTX (50 μM) does not completely inhibit the ability of the virus to form plaques, unless medium is changed several hours before the plaque assay (Figure 3.2). Proliferating BSC-40 cells, by comparison, are sensitive to 0.03 μM MTX in dialyzed serum (see Kyburz et al., 1979) and 0.5 μM in fetal calf serum that has not been dialyzed. The effect of the medium change suggested a phenomenon previously reported in mammalian cells; specifically, in medium containing serum that has not been dialyzed, MTX is considerably less cytotoxic, presumably due to the presence of thymidine in the serum (Kyburz et al.,1979). Dialysis removes metabolites that inhibit MTX toxicity. In the experiments described here, the addition of fresh medium some time before viral infection appears to have an effect similar to dialyzing serum. That is, toxicity of MTX is enhanced.

Figure 3.3A and B show an experiment carried out in medium containing dialyzed rather than undialyzed serum. Again, there is a difference in the curves between virus titrated on monolayers that had a medium change several

Figure 3.3

Effect of dialyzed serum on MTX toxicity. BSC monolayers were fed with fresh medium immediately (□) or nine hours (●) before infection with vaccinia virus. Panels A and B: dialyzed serum used to feed cells and dilute virus. Panels C and D: undialyzed serum. Panels A and C show numbers of plaques. Panels B and D express data as percentage of plaques in no MTX.

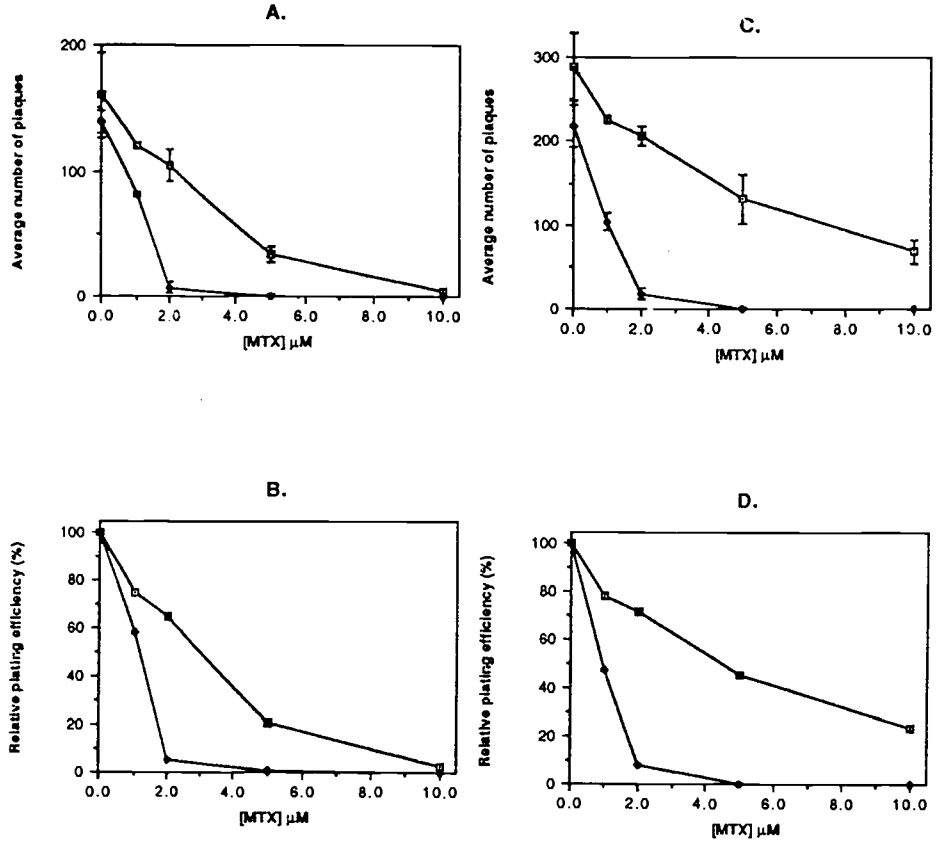
A.
C.
B.
D.
Average number of plaques
Relative plating efficiency (%)
[MTX] µM
0.0
2.0
4.0
6.0
8.0
10.0

Figure 3.3

hours before virus infection as compared with those that had not. Medium change 9 hours before infection as compared to medium change at 0 hours before infection sensitizes vaccinia virus to MTX. However, both curves go to 0 plaques. In contrast, Figure 3.3C and D show a parallel experiment in which undialyzed serum is used. (Note that the number of plaque forming units in the experiment with dialyzed serum [Figure 3.3A] is somewhat lower than that in the experiment with complete serum [Figure 3.3C].) The dialysis of the serum has rendered the virus more sensitive to MTX (cf. Kyburz et al, 1979). Even without medium change several hours before infection, 10 μM MTX can eliminate viral plaques (cf. Figure 3.3C and D and Figure 3.2). Note that in the experiment described in Figure 3.2 the highest concentration of MTX was 50 μM as compared to 10 μM in Figure 3.3.). Medium change further reduces the ability of vaccinia virus to form plaques in the presence of MTX (or of the monolayers to support plaque formation) when compared to plaque titration on monolayers that have not had medium changed several hours before infection.

Increased drug sensitivity after medium change is specific for methotrexate

In order to evaluate the possibility that the medium change had rendered the monolayers less able to support plaque formation or had caused a general susceptibility of

the virus to toxic agents, we have carried out plaque reduction curves in the presence of increasing concentrations of several drugs under two conditions: fresh medium given to cells immediately before the plaque assay and feeding of cells several hours before plaque assay. The three drugs chosen for this experiment were fluorodeoxyuridine (FUdR), which is phosphorylated to fluorodeoxyuridylate (FdUMP), an inhibitor of thymidylate synthase (Danenberg, 1977); phosphonoacetate (PAA), a DNA polymerase inhibitor (Overby et al, 1974); and isatin b-thiosemicarbazone (IBT), a specific inhibitor of vaccinia virus maturation (Easterbrook, 1962; Woodson and Joklik, 1965; Bauer, 1972; Katz et al., 1973, 1978; Cooper et al., 1979; Rada et al., 1984).

One mechanism of killing by the antineoplstic agent 5-fluorouracil is via phosphoribosyl transfer to FdUMP. The nucleoside FUdR is also activated to 5-FdUMP by cellular kinases (Ingraham et al., 1982; Tyrsted, 1984). Since FdUMP is an inhibitor of thymidylate synthase (Santi et al., 1974; Danenberg, 1977), the drug targets the same step in nucleotide biosynthesis as does MTX, although their enzyme targets differ (see Figure 3.1). Figure 3.4 shows, however, that FUdR toxicity is not enhanced by medium change prior to vaccinia virus infection of BSC-40 monolayers.

The DNA polymerase inhibitor PAA also affects nucleotide metabolism. It inhibits the final step,

Figure 3.4

Plating efficiency in FUdR with or without medium change several hours before infection. (□) Medium change at time 0. (●) Medium change at -9 hours.

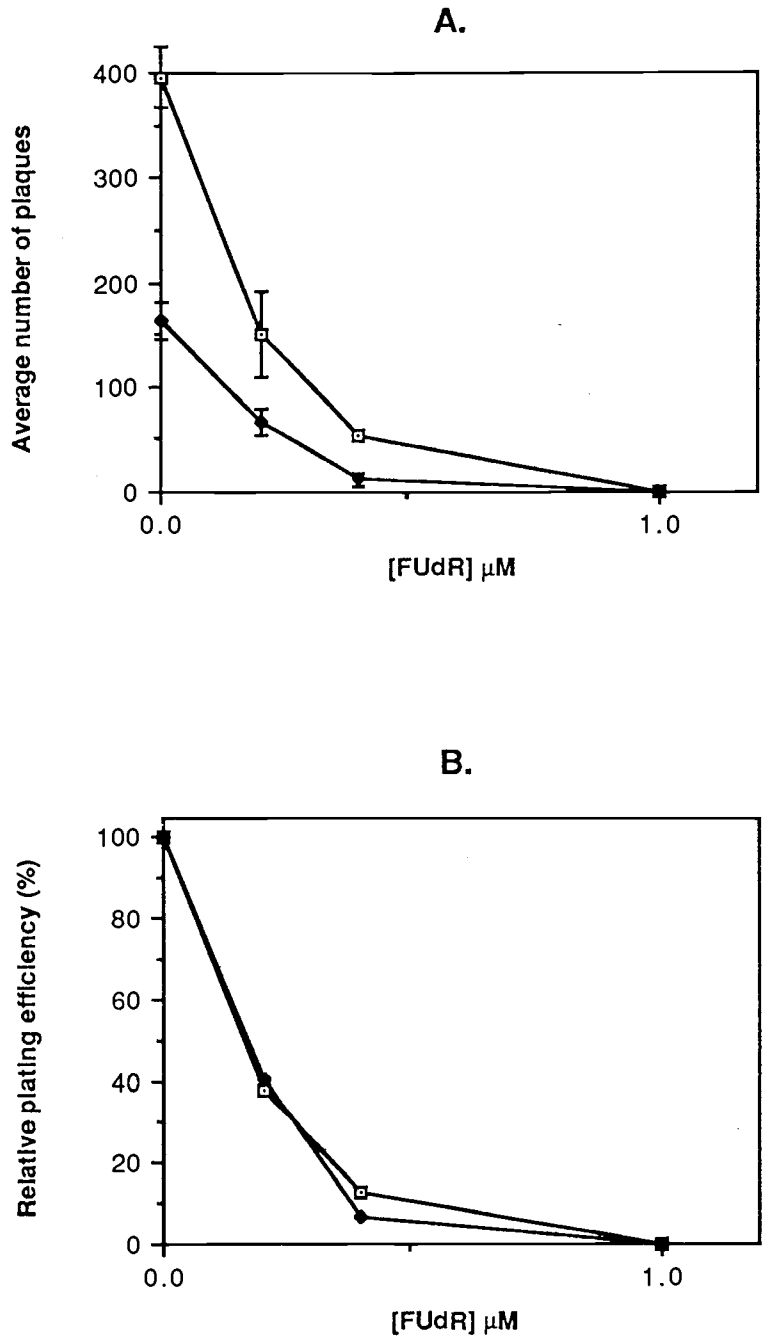
A.
Average number of plaques
400
300
200
100
0
0.0
1.0
[FUdR] μM
B.
Relative plating efficiency (%)
100
80
60
40
20
0
0.0
1.0
[FUdR] μM

Figure 3.4

incorporation into DNA. Figure 3.5 shows that the enhanced drug sensitivity observed for MTX when monolayers are fed prior to infection does not occur when PAA is used to inhibit viral replication.

IBT does not target any stage of nucleotide metabolism. Addition of IBT to infected cells at any time during the growth cycle inhibits viral maturation (Easterbrook, 1962; Bauer, 1972). IBT does not inhibit viral DNA synthesis or early transcription. However, there is a reduction in polysomes formed by late viral mRNA and a reduction in the translation of two core polypeptides (Woodson and Joklik, 1965; Katz et al., 1978; Cooper et al., 1979; Rada et al., 1984). As with FUdR and PAA, prior feeding of cells does not enhance the effect of IBT on viral replication (Figure 3.6).

The slopes of the curves in the presence of drug are not affected by the medium change. Figures 3.4B, 3.5B and 3.6B show the relative plating efficiency, with 100% representing the number of plaques in the absence of drug in each series (that is, one series without medium change several hours before and one with medium change several hours before). These results suggest that sensitivity to drugs as a result of a medium change several hours prior to plaque titration is not a general phenomenon. This effect is specific for methotrexate among the four drugs we have examined.

Figure 3.5

Plating efficiency in PAA with our without medium change several hours before infection. Symbols as in Figure 3.4.

Figure 3.6

Plating efficiency in IBT with or without medium change several hours before infection. Symbols as in Figure 3.4.

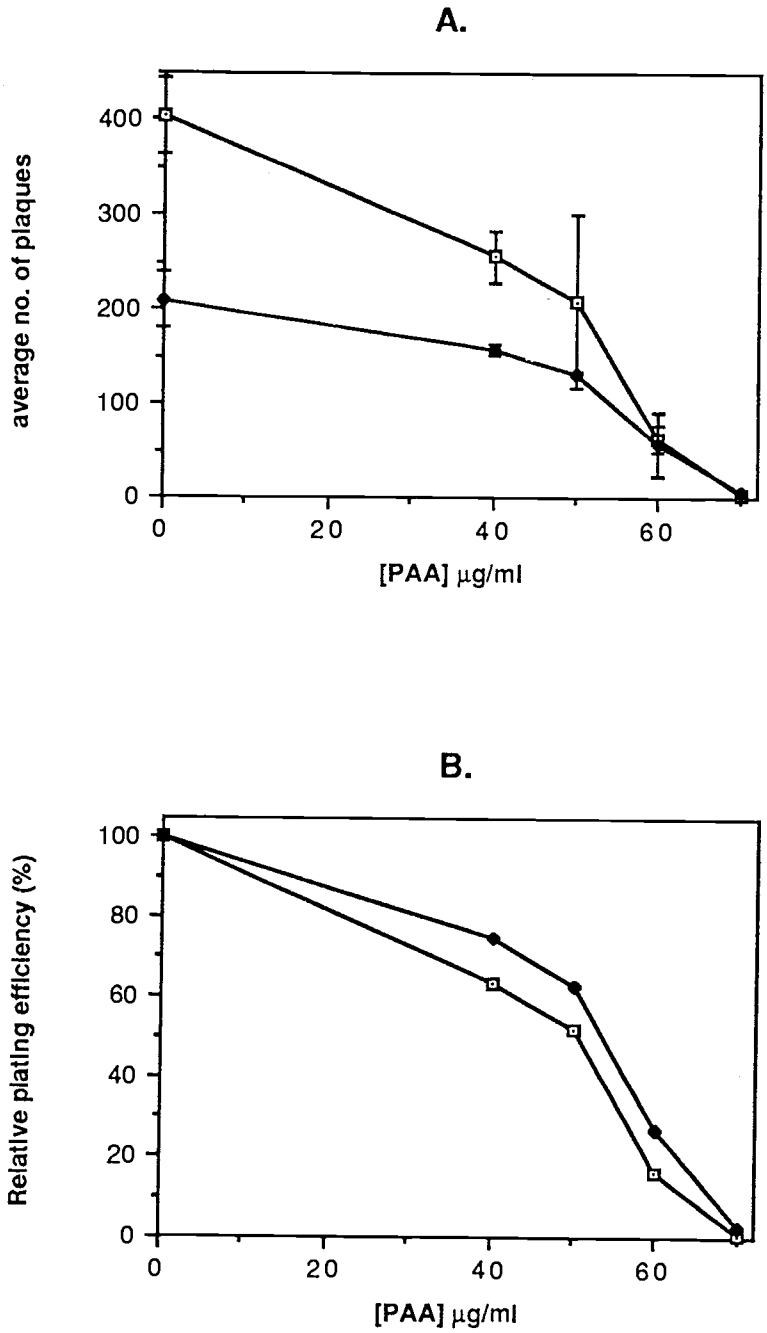
A.
average no. of plaques
400
300
200
100
0
0
20
40
60
[PAA] µg/ml
B.
Relative plating efficiency (%)
100
80
60
40
20
0
0
20
40
60
[PAA] µg/ml

Figure 3.5

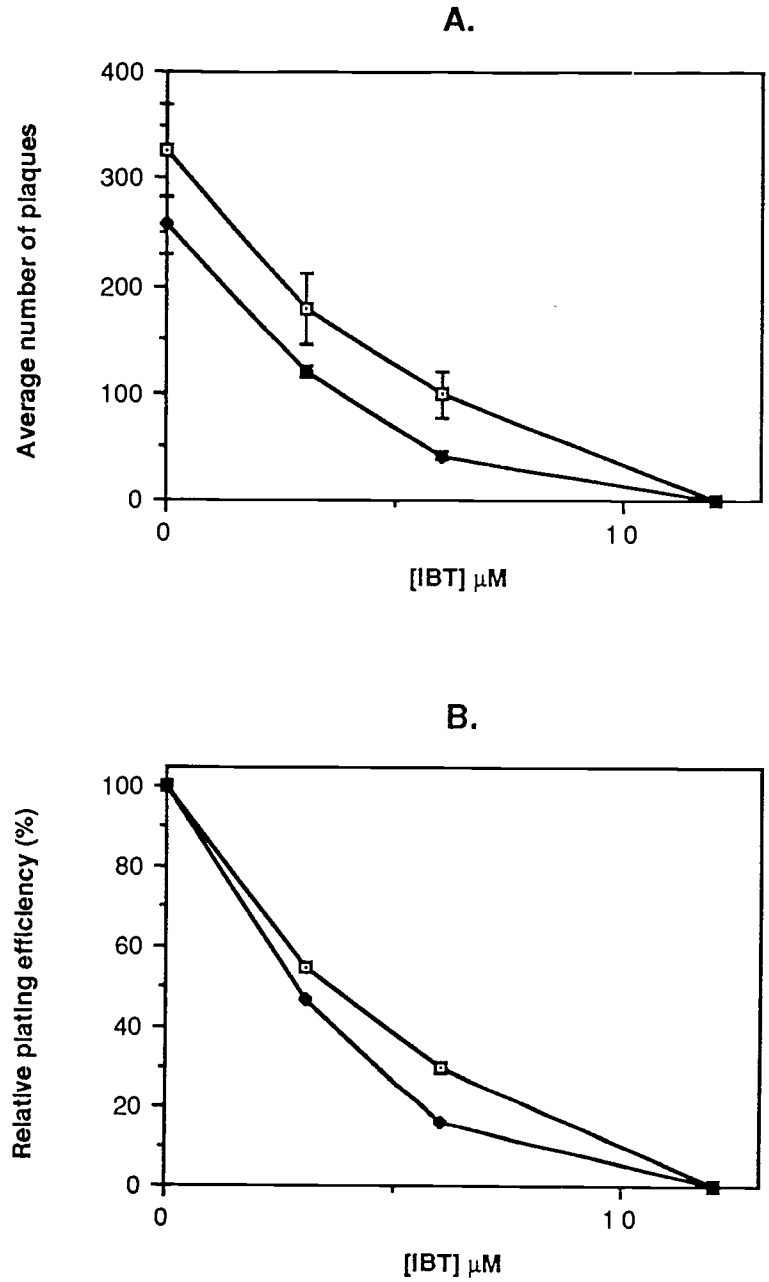
A.
Average number of plaques
400
300
200
100
0
0
10
[IBT] µM
B.
Relative platIng efficiency (%)
100
80
60
40
20
0
0
10
[IBT] µM

Figure 3.6

No viral mutants isolated during selection in the presence of MTX

In order to identify the relationship of viral and cellular functions in supplying nucleotides for viral DNA synthesis, we have tried to identify viral genetic loci that are essential to replication. To this end we have used numerous strategies for isolating virus capable of growth or plaque formation in the presence of concentrations of MTX that inhibit wild type virus. Serial passages of virus in inhibitory concentrations of MTX yielded no increase in virus resistant to MTX, either by plaque titration in the presence of the drug or by increasing titers during growth in the presence of drug (data not shown). We have tried, as well, to isolate MTX-resistant mutants from virus mutagenized with hydroxylamine and from aphidicolin-resistant virus aph^r C-10, a vaccinia virus mutant with a high spontaneous mutation rate (Spiro and Mathews, manuscript in preparation). We have isolated no MTX-resistant virus. The relative abundance of virus resistant to MTX is less than 1×10^{-9}. Similarly, passaging virus in inhibitory concentrations of FUdR have yielded no viral mutants with decreased sensitivity to that nucleoside analogue.

Dihydrofolate reductase activity of BSC 40 monolayers does not increase after medium change while thymidylate synthase

activity does increase

MTX is an inhibitor of dihydrofolate reductase. Tetrahydrofolate is oxidized to dihydrofolate during the conversion of deoxyuridylate to thymidylate by thymidylate synthase. The level of activity of thymidylate synthase is crucial to developing MTX toxicity (Moran et al, 1979). The other reactions in which tetrahydrofolate acts as a cofactor do not result in the oxidation of the tetrahydrofolate. During biosynthesis of thymidylate, however, tetrahydrofolate is oxidized to dihydrofolate. Since the reduced folate is the cofactor, purine, glycine, and methionine, as well as thymidylate biosynthetic pathways are disturbed when thymidylate synthase is active and MTX is present. We have measured the activity in BSC 40 monolayers of these two enzymes under conditions similar to those of the plaque assay, in order to see if there was some correlation between enzyme activities and the increased toxicity to methotrexate. While the results from one experiment to another were not identical (that is the levels of enzyme activity are variable from one set of experiments to another), a similar result has been seen in each experiment: dihydrofolate reductase activity does not increase over the course of the experiment while thymidylate synthase activity does increase. Figure 3.7 shows dihydrofolate reductase activity in crude cell extracts with and without medium change four hours before viral infection.

Figure 3.7

Dihydrofolate reductase acitivity in crude extracts of vaccinia virus-infected BSC-40 cells. μmol FH_2 reduced per mg of protein. (□) Medium change at 0 hours; (◆) Medium change at -9 hours. Arrow indicates time of infection.

Figure 3.8

In situ thymidylate synthase activity. Symbols as in Figure 3.7.

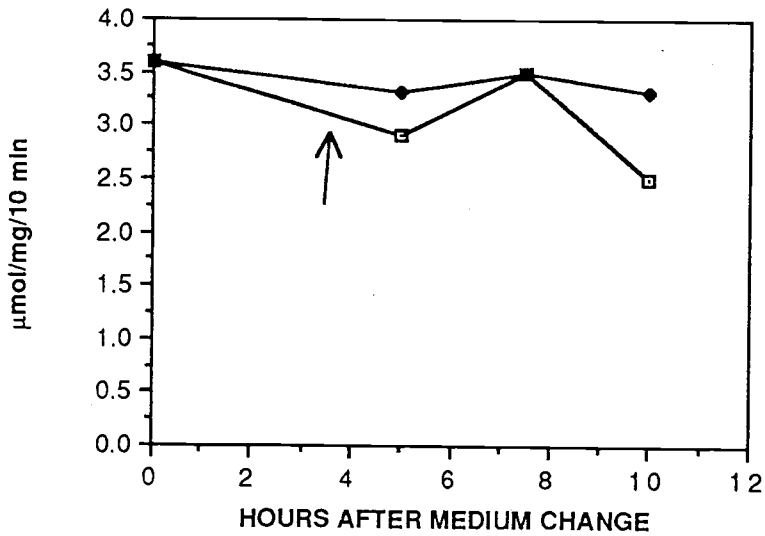
µmol/mg/10 min
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
HOURS AFTER MEDIUM CHANGE

Figure 3.7

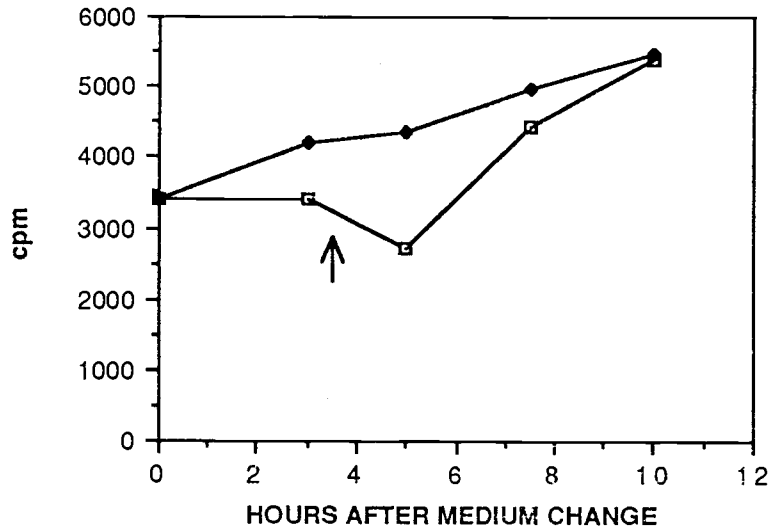
6000
5000
4000
3000
2000
1000
0
cpm
0
2
4
6
8
10
12
HOURS AFTER MEDIUM CHANGE

Figure 3.8

Figure 3.8 shows thymidylate synthase activity measured *in situ* (Rode et al., 1980).

The enzyme activity measurements suggest a mechanism for viral sensitivity following medium change: increased oxidation of folates during thymidylate biosynthesis depletes the virus-infected cells of reduced folates, which are not replenished rapidly enough to meet the needs of viral replication. The variations in enzyme activity are likely a result of variations in cell metabolism from one experiment to another; this is reflected in the variation in plaque reduction following medium change from one experiment to another as well as in variations in drug toxicity from one experiment to another (C. Spiro, unpublished; Coen et al, 1982).

The monolayers used for the plaque assays are confluent but not synchronized, according to three measurements: acridine orange staining-cytoflurometric analysis (data not shown), ^{3}H-thymidine incorporation into acid-precipitable counts (data not shown), and autoradiography of monolayers (Table 3.1). This may account for differences between these data and those of other workers on cell-cycle effects on activities of enzymes of DNA precursor metabolism (e.g. Weidemann and Johnson, 1979).

Table 3.1

Effect of medium change on number of cells synthesizing DNA.

Treatment	Positive nuclei per field*
No medium change	1.25 ±1.12
Medium change	9.90 ±3.61

*Cells were scored on the basis of incorporation of ^{3}H-thymidine and reported as the average number of positive nuclei per microscope field. Nuclei with more than eight grains were counted as positive. Twenty fields were counted. Nuclei were stained with hematoxylin.

Modulation of effects of medium change by addition of drugs prior to and during the course of infection

We have observed that when the medium on BSC-40 monolayers is changed prior to infection with vaccinia virus, there is a stimulation of BSC-40 DNA synthesis (Table 3.1). The synthesis of DNA requires de novo thymidylate biosynthesis, during which tetrahydrofolate is reduced. Virus infecting such monolayers is extremely sensitive to MTX. As discussed above, MTX treatment generally depletes cellular pools of dGTP and dTTP. The limiting supply of nucleotides can interrupt DNA synthesis leading to cell death. Depletion of reduced folate co-factors results in the increased sensitivity of the virus to methotrexate. We have tested that hypothesis by adding other nucleosides and antimetabolites prior to and at the time of infection. Such an experiment is difficult to interpret. However, we feel that it does suggest that the model of increased MTX sensitivity after medium change on the host monolayer is correct. Table 3.2 shows counts of plaques in our trial.

Results of plaque assays after five treatments are shown in Table 3.2. "No Change" indicates that a monolayer of BSC-40 cells was untreated between the time cells were plated and the time virus was added to the monolayer. "Change" indicates that at nine hours before infection the medium was replaced with fresh growth medium.

Table 3.2 Effect of exogenous metabolites on plaque formation by vaccinia virus on BSC-40 monolayers.

Treatment	No. of Plaques		Avg. @10μM MTX / Avg. control
	0μM MTX	10μM MTX	
No change	73,66	21,18	.28
Change (-9 h.)	58,61	3,3	.05
FUdR + TdR (-9 h.)	70,66	47,37	.62
TdR (-9 h.)	61,74	30,21	.38
Change (-9 h.) GdR + 8-AG (0 h.)	56,70	14,13	.21

Compounds were added at the following concentrations:
Thymidine (TdR) 100μM
Fluorodeoxyuridine (FUdR) 10 μM
Deoxyguanosine (GdR) 200 μM
8-Aminoguanosine (8-AG) 100 μM

"FUdR + TdR (Thymidine)" was included in the growth medium of one set of plates when fresh medium was added at -9 h. Such a combination should inhibit the enhanced methotrexate toxicity if our model is correct. The difficulty in interpreting data of such treatment, however, is that in the presence of FUdR + TdR, MTX is not toxic. FUdR will be phosphorylated to FdUMP and inhibit thymidylate synthase (see above). In cells that have a thymidine kinase (as do the BSC-40 cells used in these experiments), exogenous thymidine can overcome the block to de novo thymidylate biosynthesis caused by FdUMP. TdR will be phosphorylated to dTMP and supply thymine nucleotides for DNA biosynthesis. Therefore, TdR prevents the FUdR from exerting its toxic effects. FUdR in turn prevents the toxic effects of MTX. Thymidylate synthase is inhibited, so there is little oxidation of tetrahydrofolate. While MTX can inhibit DHFR, the cells have little requirement for reducing dihydrofolate (Moran et al., 1979). I have added FUdR + TdR during the period <u>before</u> infection (before addition of MTX), since that is the period of depletion of reduced folates caused by <u>cellular</u> DNA synthesis after medium change. After virus adsorption, MTX was added to the dishes. The washing of the cells at the time of infection cannot, however, assure removal of the FUdR + TdR, so their effect is not limited to the period of cellular DNA synthesis. Table 3.2 shows that this treatment resulted in more plaques than in

the cells that had no change.

The other treatments attempt to correct the deficiencies in dTTP and dGTP that can be caused by MTX treatment. 8-AG inhibits purine nucleoside phosphorylase, thus enhancing the effect of GdR on increasing levels of dGTP. The problem, again, in interpreting these results arise from the difficulty in partitioning the effects of a treatment. For example, a purine such as GdR can counter some of the toxic effects of MTX (Hryniuk, 1972; Hryniuk, 1975; Hryniuk et al., 1975) whether or not there has been a medium change, so the "rescue" might not relate to the mechanism of generating the toxicity but rather to the result of adding MTX.

DISCUSSION

The results described above may be summarized briefly as follows: vaccinia virus mutants resistant to MTX or FUdR do not readily arise by selection in either drug. Furthermore, pretreatment of monolayers with either drug renders viral replication more sensitive to the drugs in subsequent plaque titration and growth assays. Changing of the medium to fresh growth medium before infection renders the virus particularly and specifically sensitive to MTX. Medium change also reduces the ability of the cells to support viral replication in the absence of drug as measured by plaque formation.

We have asked to what extent vaccinia virus controls DNA precursor biosynthesis. Does this virus, with a large coding capacity (Moss 1985), code for nucleotide biosynthetic enzymes (in the fashion of the bacteriophage T4, which has a genome of similar size--Mathews and Slabaugh, 1986; Slabaugh et al 1984)? We have examined the effects of anti-metabolites on vaccinia virus replication and on the coupling of nucleotide metabolism and DNA replication.

MTX and FUdR are specific in their targets. MTX is a specific inhibitor of dihydrofolate reductase. Because tetrahydrofolate is oxidized to dihydrofolate only during the thymidylate synthase reaction, cells become susceptible to the effects of MTX (Moran et al., 1979) as they require

dTMP. Since the oxidized folate cofactors must be reduced to serve as donors in one-carbon reactions, the supply of purines, amino acids, and thymidylate is disrupted as a result of MTX treatment. Thus, while dihydrofolate reductase is the target of MTX and thymidylate synthase renders cells susceptible to the effects of the drug, other pathways may be most vulnerable to the effects of MTX. For example, under some experimental conditions the toxicity of MTX could be most readily overcome by supplying purines (Hryniuk, 1972; Hryniuk et al., 1975).

Three mechanisms of resistance to methotrexate, a folate analogue, are well-documented (White and Goldman, 1981; Schimke, 1984; Schimke et al., 1985): altered affinity of dihydrofolate for the analogue; amplification of the dihydrofolate reductase gene leading to a greater amount of enzyme; and reduced transport of the drug. FUdR, upon phosphorylation to fluoro-deoxyuridylate (FdUMP), is a competitive inhibitor of thymidylate synthase (Danenberg, 1977). Cellular mutants lacking thymidylate synthase, the target of FUdR, have also been described (Ayusawa et al, 1981). Alterations in enzymes that would increase the supply of substrate (dUMP) (Jackson, 1979), so that the inhibitor would be in lower effective concentration , as well as synthesis of a mutant enzyme with altered K_i could lead to resistance to FUdR (Danenberg, 1977).

We have treated virus-infected cells with inhibitors of

nucleotide metabolism and passaged the harvested virus in other cells in order to see if the continued passaging in drug might lead to mutant virus with altered sensitivity to the drugs. With respect to FUdR and MTX we have observed that the virus is about as sensitive to the drugs as are actively dividing cells. During repeated passaging of the virus in various inhibitory concentrations of the two drugs and through protocols of increasing concentrations of drugs we failed to isolate any viral mutants resistant to either of the drugs.

The inability to isolate mutants does not confirm that there are no virus-specific enzymes sensitive to the effects of these drugs. We have seen in selection of araC-resistant mutants (Spiro and Mathews, in preparation) that the choice of selection system is critical in isolating mutants; that is, simply passaging the cells in inhibitory concentrations of drug does not ensure selection of drug-resistant mutants, while in a selection system using another drug (PAA), ara C-resistant mutants were easily isolated.

The experiments described in this report show that if BSC-40 monolayers are given fresh medium several hours before plaque titration, the number of plaques formed on the monolayer is reduced. We have observed, as well, a relationship between the state of cellular metabolism and the sensitivity of plaque formation to MTX but not to other drugs that inhibit viral plaque formation. The first-

mentioned effect--reduction in the total number of plaques in the absence of drug-- varies from experiment to experiment and may suggest the role of overall cellular metabolism on viral replication. In the course of our studies, we have observed variation in titers and in sensitivity of virus to drugs from experiment to experiment.

The second effect--that is, altered sensitivity--seems to be specific, among the drugs we have tested, for methotrexate. When the plaque assay was done in the presence of MTX the number of plaques was greatly reduced in those cells that had medium change at time 0. In assays using other drugs--IBT, PAA, FUdR--no such effect was seen. FUDR is a drug that, like MTX, acts on an enzyme of DNA precursor metabolism (Danenberg, 1977; Kisliuk and Brown, 1977); PAA is an inhibitor of DNA polymerase (Overby et al, 1974); IBT interferes with viral maturation (Bauer, 1972).

Studies of the activity of the enzyme thymidylate synthase in uninfected and infected cells suggest a mechanism for this specific toxicity enhancement. Changing the medium stimulates cellular DNA synthesis and thymidylate synthase activity. There does not seem to be any increase after viral infection as compared to mock infection. The increased activity of thymidylate synthase would deplete reduced folates, which could not be replaced in the presence of the dihydrofolate reductase inhibitor methotrexate. The increased toxicity after medium change would be due to the

lower pool of folates available to the infecting virus. This could lead to a more rapid effect on biosynthesis of purines as well as of thymidylate in contrast to cells that had not been stimulated to synthesize cellular DNA prior to infection.

These results are similar to the contrasting results seen when treating cells with methotrexate in medium with dialyzed as opposed to undialyzed serum (Kyburz et al, 1979). In experiments with dialyzed serum the methotrexate is much more toxic, suggesting that the elimination of a factor in the medium increases sensitivity of the cells. In our system the stimulation of thymidylate biosynthesis in the host cells probably depletes available folates.

Our results suggest that at least in the availability of the folates that cellular and viral activities are inter-dependent. In addition we have not isolated any mutants resistant to FUdR nor to MTX, nor have we observed an increase of thymidylate synthase activity upon viral infection, suggesting that with this enzyme as well as dihydrofolate reductase, viral needs are supplied by the host enzymes. There is evidence that the virus does not recycle degraded host DNA (Parkhurst et al, 1972), so that the host enzymes of *de novo* nucleotide synthesis are critical to viral replication.

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Appendices

Appendix A. Studies on the Mechanism of Methotrexate Toxicity

Because the oxidation of tetrahydrofolate is unique to the thymidylate synthase reaction, an increase in the activity of that enzyme would likely increase toxicity of MTX by reducing the supply of available reduced folates (Moran et al., 1979; this thesis). As one approach to understanding the mechanism of action of MTX and finding methods of enhancing the efficacy of the drug as a therapeutic agent, experiments were carried out in this laboratory (Moffett, North, and Mathews, unpublished) to investigate the possibility of increasing the killing effect of MTX by using salvage pathways. Jackson (1978) demonstrated that the concentration of deoxyuridylate (dUMP), the substrate for thymidylate synthase, was the critical determinant of thymidylate synthase activity in Novikoff hepatoma cells. The addition of deoxyuridine (UdR) (see Introduction Figure 1.1), however, did not increase MTX toxicity (Moffett, North, and Mathews, unpublished; see below).

Jackson (1978) showed, as well, that deamination of dCMP (see Figure 1.1) was, at least in some cells, a more important route to dUMP than were the salvage of UdR and the dephosphorylation of dUDP. This implied that the balance between the pyrimidine DNA precursors was regulated by the

flux through dCMP deaminase as well as through ribonucleotide reductase (see also Thelander and Reichard, 1979; Robert de Saint Vincent et al., 1980; Nicander and Reichard, 1985). Moffett, North, and Mathews (unpublished) attempted to increase MTX toxicity by addition of deoxycytidine. Three results of these studies in HeLa cells were (1) that toxicity of 0.1μM MTX was decreased by the addition of 10mM deoxycytidine; (2) the intracellular concentration of dTTP apparently increased, while (3) the concentration of dCTP increased as well. Only the third of these observations is easily understood.

To further evaluate the effects of CdR on MTX toxicity, I studied the survival of mouse fibroblasts (L cells) in the presence of MTX and various nucleosides. My results (see Figure A.1) were qualitatively similar to the results in HeLa cells, although the rescue by CdR was somewhat less in L cells than that in HeLa cells.

The decrease in MTX toxicity is surprising, since, as suggested above, the increased supply of CdR would presumably increase the dUMP pool and, consequently, thymidylate biosynthesis (Jackson, 1978). Increased thymidylate synthase activity would deplete levels of reduced folates rendering the cells more sensitive to MTX (Moran et al., 1979). The increased pool of dTTP is also surprising, as depleted levels of reduced folates would reduce the ability of the cells to synthesize dTTP and lead

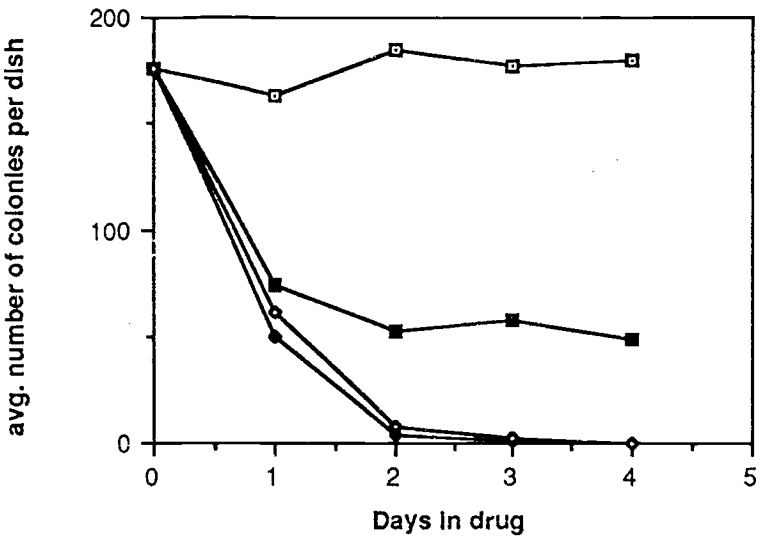

Figure A.1. Survival of mouse L cells in presence of MTX. Cells plated and grown for indicated number of days in drug(s) indicated. At that time medium replaced with fresh, drug-free medium. Cells were then grown for one week, fixed and stained. These results are representative of results obtained in numerous experiments.

- no drug
- + 0.1 μM MTX
- + 0.1 μM MTX + 0.1 mM CdR
- + 0.1 μM MTX + 1 μM TdR

to lowered dTTP pools. While a wide range of pool effects has been reported for cells treated with MTX (Adams et al., 1971; Tattersall and Harrap, 1973; Tattersall et al., 1974; Fridland, 1974; Skoog et al., 1976; Kinahan et al., 1979; Taylor et al., 1982), in general MTX decreases both dTTP and dGTP. In fact, treatment with MTX has been used as a method of inducing "thymineless" in mammalian cells (Kunz, 1982). Because the studies in our laboratory of nucleoside triphosphate (dNTP) pools were determined by an enzymatic assay (North et al., 1980), dUTP was not distinguished from dTTP. Subsequent treatment of extracts with dUTPase (a gift of Y.-C.Cheng) showed that the increased "dTTP" pool was in fact an increased dUTP + dTTP pool and the increase was due to a great expansion of dUTP levels (see Bestwick et al., 1983 and Table A.1). This was consistent with the results of others (for example, Goulian et al., 1980a). The expansion of the dUTP pool after treatment with antifolates or 5-fluorodeoxyuridine can lead to the misincorporation of uracil into DNA (Goulian et al., 1980b; Sedwick et al., 1981; Ingraham et al., 1982). It has been suggested that the repeated excision of uracil causes double strand breaks that are not repaired and, consequently, cell death. This has been proposed as a general mechanism for thymineless death (Goulian et al., 1980b; Ingraham et al., 1983). However, the values in Table A.1 for dTTP and dGTP do not indicate that the cells were depleted in dTTP or dGTP after

TABLE A.1

Effect of 0.1μM MTX on dNTP pools

Treatment	dNTP (pmol/10^6 cell)				
	dATP	dTTP	dUTP	dCTP	dGTP
None	66	64	<5	21	12
0.1 μM MTX	77	80	79	126	24
0.1 μM MTX + 100 μM CdR*	61	71	136	100	19

*see text for discussion of deoxycytidine (CdR) used in these experiments.
Pool measurements by G. Moffett.

one day's treatment. The measurement of pools at one time point may not reflect the dynamic nature of synthesis and use of DNA precursors. It is, therefore, conceivable that dTTP, for example, was transiently depleted leading to a toxic consequence. After disruption and perhaps termination of DNA synthesis, pools might have built up as the balance between synthesis of the precursors and use by DNA polymerase was shifted.

To evaluate the incorporation of dUMP into DNA after treatment with MTX, R. Bestwick (unpublished) pre-labeled cells with $^{32}PO_4$, so that each nucleotide in DNA would be labeled to equilibrium. In one experiment, he observed that there was incorporation of dUMP above background in cells treated with MTX but not with MTX plus deoxycytidine. The labeling of nucleotides with $^{32}PO_4$ eliminates the bias of added nucleosides or bases (Goulian et al., 1980b; Sedwick et al., 1981; Ingraham et al., 1982). Studies involving exogenous nucleosides and bases would be incompatible with evaluating the mechanism by which an added nucleoside (deoxycytidine) "rescued" cells from MTX. The approach of Bestwick was used in the experiments described below. The cells were treated with 1 μM MTX or with 1μM MTX + 100mM CdR and harvested after four to six hours. Nucleic acid was isolated, treated with base to hydrolyze RNA and digested with venom phosphodiesterase. The nucleotide products were separated by PEI-cellulose thin-layer chromatography (TLC)

(isobutyric acid/water/ammonia) and resolved into dGMP-dUMP, dTMP, dAMP, and dCMP. The dUMP-dGMP spot was cut out and eluted in acid. (Later experiments used a modified solvent containing isopropyl alcohol to resolve the dGMP-dUMP spot.) The dGMP-dUMP was separated by ion exchange high performance liquid chromatography (HPLC) and peaks eluting with dGMP and dUMP standards were collected and counted.

Despite improved separation of the nucleotides by alterations in TLC and HPLC protocols, the results with extracts were not consistent. In addition, the use of labeled standards at a ratio of dUMP/dGMP = .0001 indicated that even under the best separation conditions the dUMP "peak" was not distinguishable from occasional small "shoulders" of the larger dGMP peak. Figure A.2 shows the results of one experiment in which extracts of drug treated samples were processed as described above. While one sample treated with MTX shows what might be a dUMP shoulder, the duplicate MTX sample is similar to the control. The use of labeled nucleotide precursors (Goulian et al., 1980b; Sedwick et al., 1981; Ingraham et al., 1982) made separation of the nucleotides, after hydrolysis of cellular DNA, less complicated, since dUMP was more readily separated from the other pyrimidines than from dGMP.

Two other aspects of the experimental conditions are relevant. To determine incorporation into DNA after a short exposure to MTX, 100-fold higher concentrations of MTX were

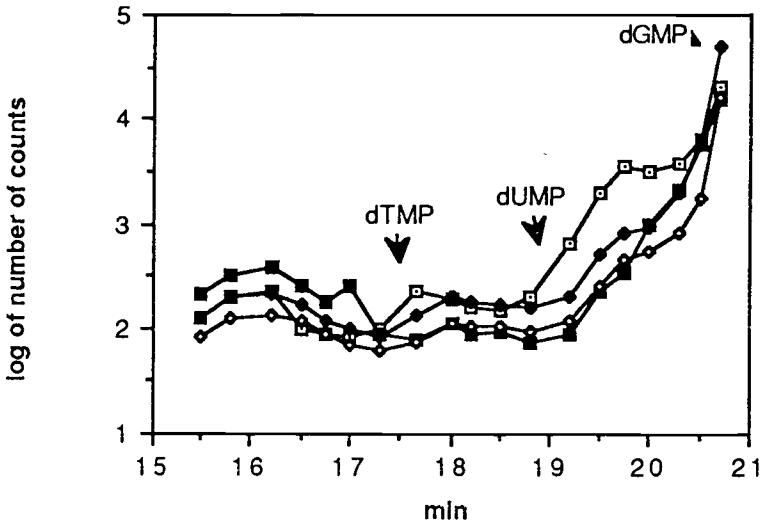

Figure A.2. Incorporation of dUMP into DNA. Radioactivity in fractions collected from HPLC column after loading dUMP/dGMP fraction from thin layer chromatography of digested cellular DNA.

used than in cell killing experiments; furthermore in Bestwick's initial experiment he used a different source for the deoxycytidine than in the initial killing curves. Killing curves using either of these alterations--that is, a different source of CdR than one specific bottle used by Moffett and by me in the earlier experiments--or higher concetrations of MTX than 0.1 μM showed no significant difference between treatment with MTX and with MTX + CdR. In other words, the rescue by CdR was only achieved with one specific lot of CdR and was effective with 0.1μM MTX or lower. The effect of DNA precursors on MTX toxicity does vary with the concentration of the drug used (see, for example, Taylor et al., 1982).

In contrast to the results of Goulian and co-workers (Goulian et al., 1980a, 1980b; Ingraham et al., 1982), we do not see a correlation between greatly expanded dUTP pools and cytotoxicity. In fact, pools were increased (as would be predicted) even more under the rescue conditions (MTX + CdR) than with MTX alone. Other values for nucleotide pools have also been reported by us (Bestwick et al., 1983). This suggests one contrast between the situation in bacterial cells and in mammalian cells. Starvation of the cells for thymidine does not cause cell death except during periods of DNA synthesis. (See Taylor et al, 1982). One explanation for the inhibition of cell death despite the enlarged dUTP pool is that perturbation of nucleotide pools by large

amounts of CdR inhibited DNA replication and, therefore, lowered flux through thymidylate synthase (cf. Rode et al, 1980, 1985; Reddy and Pardee, 1983). This contradicts the prediction of expanded dUTP as the event leading to cell death (Goulian et al., 1980b; Sedwick et al., 1981).

One area of experimentation was an attempt to identify the component in "CdR" that helped the rescue. Two lines of work (see below) led to the following conclusions: 1. the compound ,might have been the result of deterioration of CdR and did not represent another component (such as another nucleoside or a base) of DNA (the source of the CdR) contaminating the CdR preparation; and 2. other nucleosides and bases could help rescue cells from the effect of MTX (although at least in some cases the rescue was by some other means).

One approach to identifying the possible agent that helped in "CdR" rescue was to elute nucleosides and bases from columns by HPLC and oberve their elution profiles alone and in combination with other possible products of DNA hydrolysis. By reconstruction, a pattern similar to that of the rescuing "CdR" was seen when deoxycytidine and adenine were combined. In several experiments, this combination did achieve some improved rescue of methotrexate-treated cells (Table A.2). Adenine provides purines and therefore can supply depleted nuleic acid precursors (Hryniuk, 1972; Hryniuk, 1975; Hryniuk et al., 1975). In fact, various

Table A.2

Average number of colonies formed after three days' treatment of HeLA cells with drug.

TREATMENT	AVERAGE NUMBER OF COLONIES	% SURVIVAL
No drug	123	100
MTX	20	16
MTX+CR	38	31
MTX+A+CdR	102	83
MTX+"CdR"	114	93
MTX+TdR	66	54
MTX+CdR	64	52

MTX and A were used at C.1μM, CdR, CR, and TdR at 100μM. Cells were plated in the presence of drug. After three days medium was changed and the cells were fed with fresh medium without drug. Seven days later the plates were stained and colonies were counted. The averages are of three plates. "CdR" represents the lot of Calbiochemical CdR that had different properties from other preparations of CdR (from Sigma, Calbiochem, and P-L).

nucleosides gave some measure of rescue (Table A.2). Furthermore, stock solutions of deoxycytidine that originally lacked the ability to rescue MTX-treated cells with time gave HPLC elution profiles similar to that of the older, rescuing bottle. This suggested that the change in the deoxycytidine and not some other contaminant might be involved in the rescue.

Figure A.3 summarizes some of the observations made in the HeLa studies of MTX toxicity and rescue by the unusual lot of deoxycytidine. The figure shows relative measurements of cell survival, cell doubling (growth), and level of dUTP in cell extracts. Survival was measured by plating cells in the indicated drug and leaving the cell exposed to the drug for four days; growth was determined by cell counts during four days and calculating the number of doublings (for HeLa cells the generation time is about 24 hours). dUTP was measured by enzymatic assay, using the polydA/dT template, after treating the sample with dUTPase (North et al., 1980). dUTP was determined to be 87 pmol/10^6 cell in the sample treated with MTX + Calbiochem CdR. From this figure it is clear that while the cells treated with MTX + CdR survive, they do not double. Their profile is not like that of the untreated cells or like that of the cells treated with CdR alone; they survive, but they do not grow in the presence of MTX. "CdR" does not reverse the effect

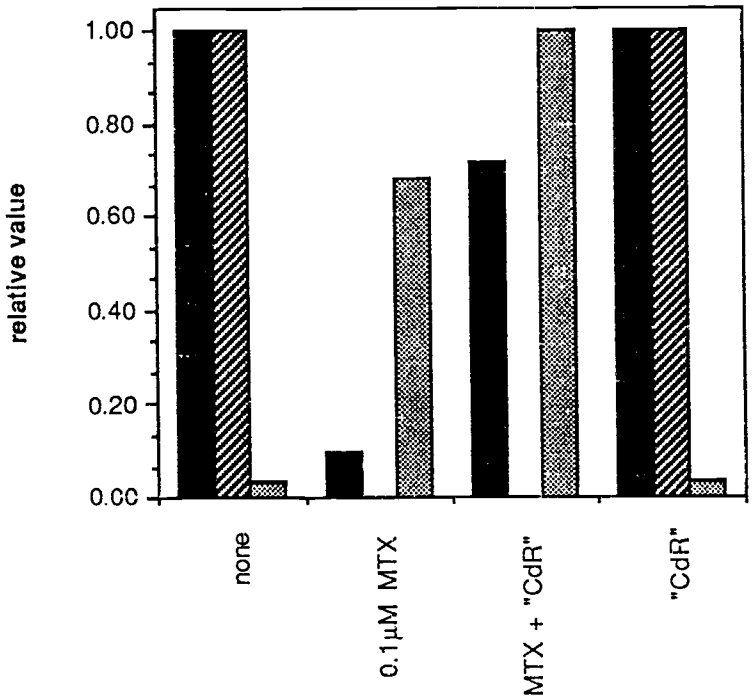

Figure A.3. Effect of MTX on survival and growth of HeLa cells and on intracellular dUTP pools. (Pool measurements by G. Moffett.)

of MTX (in contrast to the effect of hypoxanthine + thymidine in HAT medium); it allows the cell to survive despite the presence of MTX.

Though, in general, MTX-treated cells are depleted in dTTP (Adams et al., 1971; Tattersall and Harrap, 1973; Tattersall et al., 1974; Fridland, 1974; Skoog et al., 1976; Jackson, 1978; Kinahan et al., 1979; Goulian et al., 1980a; Taylor et al., 1982), MTX does not induce thyminelessness analogous to the prokaryotic model of auxotrophs deprived of exogenous thymidine. Taylor and co-workers (Taylor and Tattersall, 1981; Taylor et al., 1982) have reported that the effects on dTTP pools are drug concentration- and time-dependent. They have evaluated the ability of MTX to induce unbalanced growth (Cohen and Barner, 1954; Rueckert and Mueller, 1960; Cohen and Studzinski, 1968; Borsa and Whitmore, 1969; Frankfurt, 1981). Cohen and Barner (1954) described unbalanced growth in E. coli as the continued biosynthesis of proteins and RNA during inhibition of DNA synthesis. This led to altered protein/DNA and RNA/DNA ratios. The observation of unbalanced growth in mammalian cells treated with DNA synthesis inhibitors (Rueckert and Mueller, 1960; Cohen and Studzinski, 1968; Borsa and Whitmore, 1969; Frankfurt, 1981; Taylor and Tattersall, 1981) suggested that this syndrome might be the cause of cell death. However, the toxic effects of MTX can be observed under conditions that inhibit RNA synthesis but not

DNA synthesis, specifically, high dose MTX plus thymidine (Taylor and Tattersall, 1981). It may be the inhibition of DNA synthesis itself that causes cell death.

Studies in prokaryotes and eukaryotes have shown that transient inhibition of DNA synthesis can lead to premature reinitiation of DNA synthesis so that certain segments of DNA are replicated more than once (Billen, 1969; Pritchard and Lark, 1964; Woodcock et al., 1979; Woodcock and Cooper, 1979, 1981; Brown et al., 1983; Schimke, 1984; Mariani and Schimke, 1984; Schimke et al., 1985). Woodcock and Cooper (1981) suggested that this double replication was a general consequence of DNA synthesis inhibition and could explain toxicity of DNA synthesis inhibitors that acted on different targets. Schimke and co-workers (Brown et al., 1983; Schimke, 1984; Mariani and Schimke, 1984; Schimke et al., 1985) have demonstrated the role such endoreduplication can play in amplification of dihydrofolate reductase, which leads to resistance to methotrexate. In addition, such double replication generates highly recombinogenic free-ended, double-stranded DNA. Broken chromatids can result if only a single strand undergoes recombination (Schimke, 1984; Schimke et al., 1985). The process that the DNA synthesis inhibitor sets in motion, leading to cell death, can, in fact, give rise to a population of cells resistant to the drug.

The observations of this thesis and those in the

literature cited above suggest the complexity of the picture when certain parameters are considered. The models of Woodcock and Cooper (1981) and Schimke (1984; Schimke et al., 1985) suggest the mechanism of killing is perhaps less complex than the extensive data imply.

Appendix B. Deoxycytidylate Deaminase Activity in Vaccinia Virus-Infected Mammalian Cells

Vaccinia virus induces several enzymes involved in DNA synthesis: DNA polymerase (Moss and Cooper, 1982; Sridhar and Condit, 1983), thymidine kinase (Hruby and Ball, 1981, 1982; Weir et al., 1982; Weir and Moss, 1983), and ribonucleotide reductase (Slabaugh et al., 1984). Ribonucleotide reductase, as the first enzyme that commits metabolites to DNA biosynthesis, is critical in the de novo pathway, since nucleotides are synthesized as ribonucleotides. The other enzyme required to supply de novo deoxyribonucleotides sepecifically for DNA synthesis is thymidylate synthase. We have obtained evidence that suggests that vaccinia virus does not induce a thymidylate synthase (see above). While the irreversible deamination of deoxycytidylic acid (dCMP) to deoxyuridylate (dUMP) is not required for supplying nucleotides, the enzyme deoxycytidylate deaminase is a highly regulated allosteric enzyme (Maley and Maley, 1968; Ellims et al., 1981) that, in some cell lines studied, is apparently the major pathway supplying dUMP (Jackson, 1978; Robert de Saint Vincent et al., 1980; Nicander and Reichard, 1985). Deletion of deoxycytidylate deaminase activity can confer resistance to arabinosyl cytosine (araC) (Robert de Saint Vincent et al., 1980; Weinberg et al., 1981, 1985) by altering the balance

of pyrimidine DNA precursors. Weinberg et al. (1981, 1985) have shown that a consequence of the resulting nucleotide pool imbalance can be elevated mutation rates.

The dUMP pool determines the rate of thymidylate biosynthesis (Jackson, 1978), and it is important under conditions of stress from antimetabolites. For example, after treatment with fluorouracil (FU) or fluorodeoxyuridine (FUdR), 5-fluorodeoxyuridylate (FdUMP), a potent inhibitor of thymidylate synthase, may play a role in the antitumor effect (Danenberg, 1977; Washtien, 1984). Increased levels of dUMP can effectively compete with FdUMP, thereby ameliorating the antitumor effect of the drug. (Conversely, increased dCMP deamination would be expected to increase the toxicity of MTX, since the size of the dUMP pool affects the activity of thymidylate synthase. [see Chapter 3 and Appendix 1; Jackson, 1978; Moran et al., 1979]).

In order to determine whether dCMP deaminase was an enzyme induced by vaccinia virus in infected cells, I measured the activity of the enzyme in uninfected and virus-infected cells. The comparison of enzyme activities in uninfected vs. infected cells has been useful in determining that vaccinia virus does induce ribonucleotide reductase (Slabaugh et al, 1984; Slabaugh and Mathews, 1984). Table B.1 shows the results of two experiments in which BSC-40 cells were mock- or vaccinia virus-infected. Virus was adsorbed for fifteen minutes. Virus-containing medium was

Table B.1

Activity of dCMP deaminiase in BSC-40 cells infected with vaccinia virus. nmol/30min/mg protein

EXPERIMENT 1	
Mock-infected	21
VV-infected (moi=10)	60
EXPERIMENT 2	
Mock-infected	21
VV-infected (moi=10)	39
VV-infected (moi=20)	33

aspirated and replaced with fresh medium. Four hours later, the cells were removed from dishes by scraping with a rubber policeman. Extracts were prepared by sonication at 4° with a Kontes sonicator using the microprobe. Assays were performed essentially as described by Langelier et al. (1978).

The apparently increased activity suggested that vaccinia virus infection increased the level of acitivity of dCMP deaminase in BSC-40 cells. Similar experiments with mouse L929 cells showed, as well, that the relative level of deamination of dCMP to dUMP was higher in infected than uninfected cells. In order to find out whether the apparent increase was due to vaccinia virus induction of a viral enzyme or due to higher activity of cellular enzymes, I tested several cell lines for dCMP deaminase activity and for suitability as a host for viral infection. I obtained a V79 Chinese hamster lung fibroblast cell line (from P. Liu, University of Washington) that was lacking in dCMP deaminase (Robert de Saint Vincent and Buttin, 1980; Robert de Saint Vincent et al., 1980). This cell line supported vaccinia virus replication well (typical titers of around 3-5 x 10^8 pfu/ml, or about 0.4 the amount of virus recovered after an infection in BSC-40 cells). Table B.2 shows the level of dCMP deaminase activity in vaccinia virus-infected V79 cells. These results suggest that the virus does not have the ability to induce a unique dCMP deaminase.

Table B.2

dCMP deaminase activity in V79 hamster lung fibroblasts (nmol/30 min/mg).

EXPERIMENT 1	
Mock-infected	<1
VV-infected (moi=10)	<1
EXPERIMENT 2	
Mock-infected	<1
VV-infected	<1

These data are not, however, conclusive. For example, the experiments in V79 cells did not include a control to confirm that vaccinia virus thymidine kinase and/or ribonucleotide reductase activities were induced under the conditions of the experiment.