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RECENT ADVANCES IN CHEMOTHERAPY OF VIRAL DISEASES[1]

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I. INTRODUCTION

The recent impact of molecular biology is being felt in a wide variety of disciplines, including virology. Within the past year 2 symposia have been devoted exclusively to problems in virology: one to antiviral substances and the other to the molecular biology of viruses. Within the past 2 years several excellent reviews have appeared that describe the progress made in our understanding of the mechanisms not only of viral reproduction, but also of viral chemotherapy (69, 84, 88, 127, 156, 179, 181, 195, 224, 265, 337, 343).

An understanding of the biochemistry of viral replication should provide a basis for the design of drugs that have selective actions on viral reproduction. Compounds in vast number, both synthetic and derived from natural sources, have exhibited antiviral activity. Of these, idoxuridine (IUdR, 5-iodo-2′-deoxyuridine) (Section IV) and amantadine (adamantanamine, Symmetrel) (Section VII) have achieved the status of drugs permitted by the Food and Drug Administration to be sold in the United States, and several other agents are under clinical investigation.

As our knowledge of the molecular basis of viral reproduction becomes more sophisticated, because of applications of modern techniques of biochemistry and molecular biology, the goals of the chemotherapist in the design of potential antiviral substances have been given emphasis. Thus, we now know of several biochemical events that are unique to the virus-infected cell or to the reproduction of the virus, and these should lead to the development of new potential antiviral agents.

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Animal and bacterial viruses have many similarities in morphology and biochemistry; but the differences that make each virus a distinct entity preclude the universal effectiveness of a single antiviral agent. Accordingly, it is imperative to investigate the uniqueness of each virus, as well as the properties that are common to viruses as a group, in order to design or to select intelligently a potential antiviral agent.

Animal, plant or bacterial viruses may be divided into 2 main types distinguished by whether their nucleic acid component is RNA or DNA. All the genetic information required for viral reproduction resides in the nucleic acid component, since infectivity and subsequent formation of a complete viral particle can occur when the highly purified nucleic acid derived from some viruses is incubated with the appropriate host mammalian or bacterial cells. The newly formed viral particle is identical in all respects to that produced when the host cell is infected with the complete viral particle from which the infectious nucleic acid was derived. Thus, the RNA or DNA component of the virus not only can replicate itself but also carries the information to induce the formation of those new enzymes that are required for the synthesis of the progeny nucleic acid, as well as of the specific coat protein.

Many of the enzymes induced by virus infection catalyze reactions that are vital for the replication not only of the virus, but also of the host cell. There may be good biochemical explanations for this wasteful phenomenon but, if so, these require a greater degree of understanding than we now possess. The viral particle does not contain the enzymes required for its own reproduction; rather, after penetration into the host cell, it either uses enzymes already present or induces new enzymes for its replication. The induced enzymes appear to be qualitatively distinct in that they may have different chemical and physical properties. In some cases the virus-induced enzyme can be separated physically from the corresponding enzyme present previously in the host cell; thus, a possibility exists for selective inhibition of viral reproduction. Although, as will be discussed below, an attempt was made with one antiviral agent, IUdR, to seek such a difference between the host and virus-induced enzyme with respect to susceptibility to inhibition, none was observed. In spite of the failure to detect a difference in this case, it is still possible that this approach will be successful in other cases.

It should be possible to take advantage of the fact that highly specific enzymes and proteins are synthesized during the normal replication of a virus. In bacterial chemotherapy, penicillin interferes with bacterial cell wall synthesis and streptomycin with bacterial protein synthesis. Agents may be found that can interfere specifically with the synthesis and assembly of virus coat protein, a component unique and essential to the virus.

An enzyme is synthesized, presumably in the virus-infected animal cells, which permits release of newly formed virus particles from the host cell. Does the synthesis or the catalytic activity of this essential enzyme not afford an area for specific drug attack?

Both RNA and DNA may contain small amounts of bases other than adenine,

thymine, guanine and cytosine. Only a few of the animal virus nucleic acids have been examined in sufficient detail to detect unusual purine or pyrimidine bases. The methylated bases in transfer-RNA are involved in directing the amino acid for messenger-RNA recognition in protein synthesis, but the function of methylated bases in phage DNA has not been elucidated. Winocour *et al.* (368) found the DNA of polyoma virus to contain 5-methylcytosine in amounts about 0.1 that present in mammalian cell DNA as typified by mouse kidney. The significance of this base is unknown. No effect on plaque-forming ability was observed when this viral DNA was hypermethylated enzymically to contain 8 additional methyl groups per DNA polymer. Further studies will enable better judgement of the probabilities of successful antiviral attack in this direction.

One of the most promising areas being investigated in several laboratories is the mechanism of viral nucleic acid replication. Host cell RNA is derived by transcription of host cell DNA, whereas viral RNA must be transcribed from viral RNA with the aid of an RNA polymerase (synthetase, replicase) induced by the virus. The virus-induced RNA polymerase is relatively unstable (19). Haruna *et al.* (146–149) and Pace and Spiegelman (267) have isolated this enzyme recently in highly purified form from bacteria infected with the RNA bacteriophage, QB, and have demonstrated unequivocally the ability of this enzyme to synthesize infectious RNA *in vitro*. These brilliant accomplishments permit investigations of the effect of drugs on an enzyme that is unique to the virus. Haruna and Spiegelman (149) have indeed initiated such investigations and demonstrated that specific polyribonucleotides do interfere selectively with the formation of bacteriophage ribonucleic acid *in vitro*. It is to be anticipated that similar studies with an animal RNA-virus will be explored in the near future.

A similar demonstration with virus-specific DNA polymerase has not yet been accomplished, but it is reasonable to predict that various technical difficulties eventually will be overcome. Such accomplishments with enzymes unique to animal RNA- and DNA-viruses should result in test systems that will enable rapid evaluation of potential antiviral agents.

Since the number of potentially antiviral substances that have been investigated is so large, comments will be restricted to compounds that either are in clinical use or trial or appear to be of particular interest. The viral induction of enzymes and of malignancy will be considered first because the former provides a virus-specific site of attack by antiviral agents and the latter indicates an area of potential importance in the application of these agents.

II. ENZYME INDUCTION

Evidence for the "induction" of enzyme activity in cells infected with various animal viruses has been well documented in a number of different laboratories. The specific enzymes induced vary with the infecting virus and include RNA-dependent RNA polymerase, DNA polymerase, thymidine kinase, aspartate transcarbamylase, alkaline deoxyribonuclease, acid deoxyribonuclease, "neutral" deoxyribonuclease, thymidylate synthetase, dihydrofolic acid reductase, deoxycytidylate deaminase, uridine kinase, and arginase (90, 104, 113, 145, 150, 166,

202–204, 209–212, 243, 260, 272, 280, 309, 342; additional references cited in 179). There is general agreement that a genuine increase in enzyme activity occurs, but the mechanism for this biological phenomenon has not been clarified. Many factors must be considered before its control can be regarded as analogous to a genetic one. Let us consider the enzyme thymidine kinase, the activity of which is increased after infection of the host cell with such viruses as herpes simplex, vaccinia, polyoma and pseudorabies. An initial increase in this enzyme is not essential for the biosynthesis of viral DNA *via* the *de novo* pathway, as this pathway provides deoxythymidylic acid by the reaction catalyzed by thymidylate synthetase. Thymidine kinase has been considered to be a scavenger enzyme, in that it permits re-utilization of thymidine liberated during the degradation of DNA. Kit and his colleagues (209) have suggested that this enzyme may serve a useful function in infected cells as it affords the replicating DNA virus an ample supply of the essential precursor, deoxythymidylate. This may be accomplished by the following possible mechanisms: (1) counteraction of the catabolic effects of phosphatase cleavage of deoxyuridylate and deoxythymidylate by phosphorylation of deoxyuridine and thymidine restores, respectively, the pools of deoxyuridylate needed for the thymidylate synthetase reaction and of deoxythymidylate, the product of this reaction; (2) prevention of the depletion of the pools of deoxythymidylate could have the effect of activating or stabilizing deoxythymidylate-kinase; (3) phosphorylation of circulating thymidine, derived either exogenously or from degraded cellular DNA, would result in the formation of additional deoxythymidylate.

The increase in enzymic activity that follows viral infection could be the result of one of several possibilities. (1) The formation of new enzyme *via* synthesis. (2) Inhibition or inactivation of either a repressor or an inhibitor present in the uninfected host cell. Thus, the increase in aspartate transcarbamylase observed by Consigli and Ginsberg (63) in cells infected with adenovirus type 5 reflects the depletion of deoxyribonucleoside triphosphates that normally exert a feedback inhibition in uninfected cells. (3) The introduction of an activator or substrate. Thymidine kinase is an excellent example of an enzyme the activity of which is dependent upon an number of factors. Okazaki and Kornberg (264) have studied the complexities of this enzyme derived from *Escherichia coli* and have identified specific activators and inhibitors. Either high levels of ATP or low levels of ATP plus deoxycytidine diphosphate activate thymidine kinase (264), while deoxythymidine triphosphate inhibits this enzyme (40, 174). The presence of deoxycytidine diphosphate, an activator, results in a greater inhibitory effect of deoxythymidine triphosphate on thymidine kinase (264), and deoxyadenosine triphosphate and deoxyguanosine triphosphate serve not only as phosphate donors, but also as activators. Thus, the situation in the intact cell is complicated indeed. The phenomenon of activation presumably reflects a conformational change in the enzyme that is responsible for increased activity. The milieu created by infection with a virus could result in an allosteric activation of the host cell enzyme. The altered properties of the "induced" enzyme, such as altered heat stability, Michaelis constant, chromatographic behavior, or sero-

logical behavior, could represent merely a conformational or allosteric modification of the host cell enzyme present normally.

Formation of "induced" enzymes may be prevented by the use of inhibitors of protein synthesis (*e.g.*, *p*-fluorophenylalanine or puromycin), but this does not prove induction, because the maintenance of thymidine kinase in an active form requires the presence of activators, the formation or maintenance of which may depend on a continued supply of a labile protein.

Jungwirth and Joklik (186) have pointed out that even evidence for the synthesis of enzymes not present normally in the host cell does not prove that the viral genome is involved, since both interferon (Section VI) and the "uncoating protein" are not detected in uninfected cells, but appear to be coded for their amino acid sequence in host cell DNA that is normally repressed in the absence of infection.

Proof that the increased enzyme activity is a result of transcription of the viral DNA and subsequent translation of the formed messenger-RNA would receive strong support by the demonstration of either a different amino acid sequence or a different composition of the "induced" enzyme when compared to that present in the uninfected cell. Unequivocal proof of viral genome induction of enzyme activity will require this synthesis to occur in a cell-free system that is free of other genetic information (179).

An increase in enzyme activity is observed not only upon infection of mammalian cells with certain viruses, but also when cells either enter their S-1 phase of growth or are exposed to various inhibitors of DNA synthesis: IUdR, 5-bromo-2′-deoxyuridine (BUdR), cytosine arabinoside, mitomycin C, or amethopterin (210, 213, 338).

The presence of IUdR or BUdR in virus-infected cells has been shown by Kaplan *et al.* (191) to affect the activity of enzymes concerned with DNA formation. Normally in cells infected with pseudorabies, the increase in enzymic activity stops 6 hours after infection and enzyme activity is low after 12 hours, but in the cultures treated with these halogenated deoxyribonucleosides, DNA-synthesizing activity was found for up to 12 hours. Kit *et al.* (210) found the effects of cytosine arabinoside and SV40 virus on the induction of thymidine kinase to be additive.

McAuslan (241, 242) had shown that inhibitors of the synthesis of DNA had no effect on enzyme formation but prevented the normal stoppage of the synthesis of thymidine kinase in cells infected with poxvirus. The thymidine kinase activity of cultured cells derived from human liver increased when cellular reproduction was inhibited by either amethopterin or 5-fluorodeoxyuridine (FUdR), and this increase in enzyme activity was prevented by thymidine (86). Jungwirth and Joklik (186) found an increase of the activities of DNA polymerase, deoxyribonuclease, and thymidine kinase beginning about 90 minutes after infection of HeLa cells with vaccinia virus and ending 4 hours later; however, when the synthesis of viral DNA was inhibited, the "switch off" mechanism for these 3 enzymes failed to function. Sheinin (317) found the specific activity of thymidine kinase to increase about 2 hours before the onset of synthesis of polyoma virus-

DNA in infected cells of the mouse embryo. The Michaelis constant, substrate inhibition, and thermal stability of the induced enzyme was different from that of the uninfected cell. Infection with polyoma virus increased the activity of thymidine kinase, thymidylate kinase, thymidylate synthetase, deoxycytidylate deaminase, DNA polymerase, and dihydrofolate reductase, but not of uridine kinase, thymidylate phosphatase, deoxyuridylate phosphatase, deoxycytidylate kinase or deoxyadenylate kinase (209). The increase in thymidine kinase activity produced by SV40 virus was prevented by puromycin, *p*-fluorophenylalanine or dactinomycin (actinomycin D) (209, 210).

DNA polymerase is induced by vaccinia (129, 237), herpes simplex (292), pseudorabies (260) and polyoma viruses (209); the properties of the DNA polymerase induced by herpes simplex virus are different from those of the enzyme of the host cells (203).

Enzyme induction is not confined to infection by DNA viruses. RNA-dependent RNA nucleotidyl transferase is induced upon infection with several RNA viruses (influenza virus, polio virus, and mengo virus) (8, 18, 19, 146, 359) and the induction of this enzyme by influenza virus is prevented by dactinomycin, a compound known to inhibit DNA-dependent RNA nucleotidyl-transferase. Dactinomycin has this effect only in the early stages of the replication of influenza virus. Among the RNA viruses dactinomycin influences not only influenza virus, but also reovirus, Rous sarcoma virus, fowl plague virus, and vesicular stomatitis virus (22, 101, 123, 124, 345, 364, 366).

Dactinomycin has no inhibitory effect on the replication of many other RNA viruses, *e.g.*, polio virus, mengo virus, Newcastle disease virus, and encephalomyocarditis virus (151, 166, 294). RNA polymerases resistant to dactinomycin have been observed in these viruses (18, 19). The inhibition by dactinomycin of avian myeloblastosis virus varies with the host cell infected, this virus being resistant to inhibition when grown in myeloblasts (1, 346). Low doses of dactinomycin may stimulate the replication of measles virus by inhibition of the production of interferon (2).

In a series of elegant experiments Haruna and Spiegelman (147, 148) and Spiegelman *et al.* (330) purified 2 phage RNA-dependent RNA polymerases that functioned only with the RNA genome of its origin and established the synthesis *in vitro* of infectious RNA of the same molecular weight as that derived from the intact virus.

Gibbard *et al.* (120) observed that infection of the chorioallantoic membrane of the chick embryo with Rous sarcoma virus increased uridine kinase activity 10-fold, and Rada and Gregusova (285) found an increased phosphorylation of 6-azauridine in cells infected with Rous sarcoma virus.

III. ONCOGENIC VIRUSES

The role of exogenous viruses (polyoma virus, SV40 virus, adenovirus type 7, 12, or 18) and of the activation of endogenous viruses by irradiation, in the production of some neoplasms in a wide variety of animal species is well established, and these discoveries introduce a new and potentially important parameter in the

study of antiviral agents. Whether oncogenic viruses have a role in the etiology of neoplasia in man has not been established, but it would be strange indeed, in view of the induction of neoplasms by viruses in other species, if this were not the case. Nevertheless, acceptance of this role of viruses in man requires evidence. Several interesting reviews of tumor viruses and tumor antigens have appeared recently (34, 76, 135, 214).

Several viruses (Rous sarcoma virus, polyoma virus, SV40 virus, adenovirus) can either replicate in certain cells (and so destroy them), or produce transformation of the cell. There is no evidence for the persistence of DNA viruses *per se* in transformed cells. Such cells are genetically altered and have acquired a set of new characteristics that include loss of contact inhibition, acquisition of new antigens, and alteration of chromosomes either in number or in appearance.

It has been postulated that at least a portion of the virus DNA is incorporated into the genetic apparatus of the transformed cell, presumably in a manner resembling lysogenic phage. Supporting evidence was provided by Axelrod *et al.* (9), using the DNA-agar technique described by Bolton and McCarthy (37); Black and Rowe showed that the DNA of polyoma virus can hybridize with a portion of the DNA derived from the transformed cell (35). In addition, they demonstrated specific binding (complementary base sequences) between fragments of polyoma virus DNA and normal DNA of several mammalian species, including man.

Winocour (367), using the technique of nitrocellulose membrane filtration, confirmed these results in principle by demonstrating hybridization between radioactive RNA, derived by enzymic synthesis with normal mouse DNA as a primer for the DNA-dependent RNA polymerase, and DNA from mouse tumor cells induced by polyoma virus and free of virus. There was, however, *no* increased hybridization between the virus DNA and RNA synthesized with DNA primer derived from a tumor cell induced by polyoma virus. Benjamin (29) presented evidence for the persistence of a viral genome in cells transformed by polyoma virus by showing that a small fraction of pulse-labeled RNA derived from these virus-free cells could hybridize with polyoma DNA. No cross hybridization was observed between viral DNA and RNA derived from normal or spontaneously formed malignant cells. Reich *et al.* (293) presented evidence also that SV40 DNA is present in the DNA of cells transformed by SV40 virus, and that this DNA is responsible for the production of the SV40-specific T-antigen. Additional evidence that at least part of the virus DNA is in the transformed cell is provided by the presence there of a new antigen that is specific for the virus that caused the transformation (121, 134, 136, 137, 289, 305, 322). Black and Rowe (35) produced SV40 T-antigen by infection of cells with the DNA of SV40 virus and thereby supported the contention that the occurrence of this antigen in transformed cells is a result of the presence of at least this portion of the SV40 genome. Gerber (115) induced SV40 virus in a culture of hamster ependymoma, produced originally by SV40 virus, by treatment with proflavin, hydrogen peroxide or mitomycin C. Sabin (304) observed the formation of antigen not to be dependent on the synthesis of virus DNA; but dactinomycin

at toxic levels partly depressed, and streptovitacin A, a hydroxylated analog of cycloheximide, completely suppressed the synthesis of both tumor and virus antigens. Fujinaga and Green (108) investigated the mechanism of viral carcinogenesis and observed a virus-specific RNA associated with the polyribosomes of tumors and of transformed cells induced by adenovirus type 12. One part of this RNA was present in 20 to 50 of the total messenger RNA, whereas one part of viral DNA was present in 10,000 parts of cell DNA. Interferon administered before infection with SV40 virus delayed the formation of T-antigen (266). The effect of various pyrimidine analogs on the formation of T-antigen has been reviewed by Melnick and Rapp (247). Cytosine arabinoside, IUdR or 5-fluorouracil neither depresses nor delays its formation (41, 247), but cytosine arabinoside can prevent the formation of virus antigen (44, 163, 247). Maass and Haas (234) observed similarly that IUdR inhibited the replication of SV40 virus but not the formation of SV40 antigen.

RNA viruses that produce certain naturally occurring leukemias of fowl, in contrast to the oncogenic DNA-viruses, are not only in the neoplastic cells with which they replicate, but also in other cells of the fowl. The tumor RNA-viruses are present in tumors derived from naturally occurring tumors, as well as in cells transformed to malignancy *in vitro*. The cells transformed by Rous sarcoma virus produce infectious virus only when Rous-associated virus, a herpes virus, is present; the latter is required to code for the coat proteins. The adenovirus type 12 can cause in man a mild upper respiratory infection, but when the newborn hamster, rat or mouse is inoculated with a sufficient dose of this agent, neoplasia is produced. There is no evidence for the production of cancer by this virus in man.

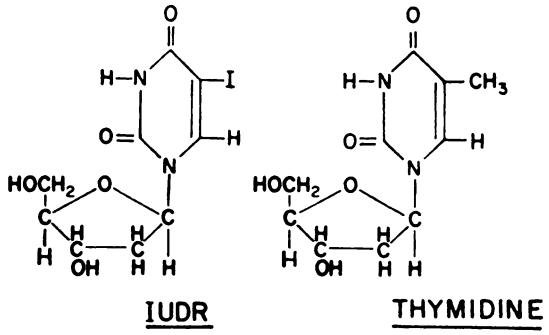
Purified DNA from polyoma, SV40 and Shope papilloma viruses and purified RNA from mouse leukemia virus produce tumors (71, 114, 173, 239), but a high dose is needed (10^8 or 10^9 DNA molecules for each transformation) (67). Hays and Alpert (152) found RNA from neoplastic tissue produced in mice by infection with Gross or Rauscher leukemia virus to be inactive whereas cell-free extracts produced the specific disease associated with each virus.

The induction of tumors by adenovirus type 12 and of polyoma virus in newborn hamsters has been markedly reduced by the administration of IUdR (97, 158) and tumors induced by polyoma virus are affected similarly by 5-iodo-2′-deoxycytidine (97). Thus, the induction of neoplasia by oncogenic viruses can be markedly inhibited. If viruses were found to be related to neoplasia in man, a possibility might be afforded not only of very early prophylactic therapy with specific antiviral agents, but also of prevention by vaccines similar to those that have been shown to antagonize virus-induced leukemias of mice.

IV. HALOGENATED PYRIMIDINEDEOXYRIBONUCLEOSIDES

A. Biological properties

The antiviral activity of IUdR and BUdR has been established in cell culture, in animals and in man. After the report by Herrmann (156a) that IUdR and



BUdR inhibit the replication of several DNA-containing viruses *in vitro*, particularly vaccinia and herpes simplex, Kaufman and his collaborators (194, 199, 200), by means of solutions of either IUdR or BUdR applied frequently to the cornea, suppressed the keratitis caused by these 2 viruses in rabbits and, more importantly, suppressed herpes simplex of the corneal epithelium in man. The studies in rabbits were confirmed and extended to other derivatives of IUdR (268). Kaufman (195) has presented the results of 5 independent double-blind studies that confirm the efficacy of IUdR in acute dendritic keratitis in man.

Although the replication of DNA-viruses (*e.g.*, herpes simplex, vaccinia, pseudorabies, adenovirus, and polyoma) is inhibited by IUdR, RNA-viruses are not affected with the exception of Columbia-SK encephalitis (100) and Rous sarcoma virus (101), for which special reasons exist. The synthesis of DNA is required for the replication of Rous sarcoma virus (11, 12, 347), and this requirement occurs only during the early stages of the reproduction of this virus (13, 14). This DNA appears to be cellular in nature. Inhibition of the multiplication of Rous sarcoma virus occurs only when IUdR is administered to cells at the time of infection (101). Replication of this RNA virus is inhibited also by dactinomycin, an inhibitor of DNA-dependent RNA synthesis (345), mitomycin C, BUdR and amethopterin (12). Since IUdR had no effect on the growth of influenza virus in these cells, Bader (12) concluded that the inhibition by IUdR was not related to an effect on the host cells. In fact, cells pretreated with BUdR for 3 or 4 days and infected with Rous sarcoma virus after removal of the analog from the medium supported the replication of the virus as satisfactorily as did untreated cells (11).

Similarly, in mouse embryo cells that had been exposed to IUdR (10 μg/ml) for as long as 69 hours before infection with polyoma virus, and then transferred to a medium free of drug, infectious virus was formed in normal or even greater-than-normal amounts (254). This concentration of IUdR inhibited irreversibly the replication of the mouse embryo cells and resulted in enlarged cells which permitted the formation of increased amounts of virus. Dunnebacke and Reaume (78) had observed that the yield of polio virus particles is directly related to the size of cells. Kimura and Mori (205) observed that IUdR inhibited the replication of polyoma virus when grown in a strain of mouse cells sensitive to IUdR but not

in a subline of these cells that was resistant to IUdR. These results are in contrast to the observation of Dubbs and Kit (74, 75) that reproduction of vaccinia virus and herpes simplex virus in a cell line deficient in thymidine kinase is inhibited by BUdR because of the ability of these viruses to induce thymidine kinase. Since polyoma virus can induce thymidine kinase (77, 103, 145, 209, 317) also, the report of Kimura and Mori (205) is rather surprising. Their work implies that the inhibition produced by IUdR is directed at a mechanism vital to the cell rather than to multiplication of viruses.

The problem of whether IUdR is a specific antiviral agent or exerts its effect by producing cell toxicity was investigated by Cramer *et al.* (66). They found inhibition of herpes simplex virus at a concentration 0.1 that required to inhibit cell reproduction. Similarly IUdR, diacetyl IUdR and BUdR prevented the formation of infectious herpes simplex virus in rabbit cornea in concentrations that did *not* affect the synthesis of DNA by the normal basal epithelial cells of the cornea (141). The free pyrimidine base 5-iodouracil neither prevented viral reproduction nor affected the synthesis of DNA (141).

Numerous reports have confirmed the efficacy of IUdR in the treatment of herpes simplex infections of the corneal epithelium in man, although its effectiveness in stromal disease is doubtful. Hanna and Wilkenson (143) observed that IUdR inhibits incorporation of radio-thymidine into the corneal epithelium of rabbits infected with herpes simplex virus, but this effect is not seen in uninfected corneal tissues. IUdR inhibits cell regeneration of uninfected corneal tissues when they are experimentally injured but not in "normal" tissue infected with DNA-virus (315).

The results of studies of topical therapy with IUdR of herpes simplex of the eyelids, lips, and cheeks in man have been contradictory, the problem presumably being one of insuring penetration of the drug into the infected cells. IUdR (0.1%) in aqueous solution was applied in a double-blind trial with a modified air gun in an attempt to achieve an intradermal injection, and the claim was made that a therapeutic effect on cutaneous herpes was indeed achieved (185). Shell (318) stressed that both the dosage regimen and the vehicle used to dissolve IUdR are critical to the amount of the drug absorbed by rabbit skin; the most effective solution tested was 0.1% IUdR in propylene glycol-water, 75:25. It is curious that higher concentrations of IUdR appeared to be less effective. Goldman and Kitzmiller (122) similarly stressed the importance of the vehicle for IUdR and found a 1% solution of IUdR in dimethylsulfoxide-water 90:10 to suppress herpes simplex of the skin. Mild irritation was the only adverse reaction. MacCallum and Juel-Jensen (236) treated herpes simplex of the skin in man by application of a 5% solution of IUdR in undiluted dimethylsulfoxide 3 times a day for 3 days in a total volume of 1 ml. A 63% reduction in duration of lesions was observed, significantly higher than the 43% reduction in the patients treated with the solvent alone. Of importance is that no recurrences occurred in the *same* site in patients treated with IUdR, in contrast to recurrence in all patients treated with dimethylsulfoxide alone.

Recurrent cutaneous herpes lesions in 111 patients treated with IUdR showed

a decreased healing time from a mean of 12.5 to 5.7 days (64); the investigators also stressed the importance of the proper selection of the vehicle for IUdR, since the drug must penetrate in adequate concentration to the deeper epidermal cells, where the herpes simplex virus infection and replication occur. In addition, the best results were obtained when solutions of IUdR (0.1%) in 1.4% polyvinyl alcohol were applied for a total of 4 days, beginning at the first prodromal symptom and applied every 5 minutes for the first hour, then every hour for 12 hours, followed by every 2 hours except when asleep. Jawetz *et al.* (178) treated experimental keratitis in rabbits with a 0.9% solution of IUdR in 25% aqueous polyethylene glycol and although the herpes simplex virus content was reduced by 99 to 99.99%, complete eradication did not occur. Virus could be found for a longer period of time in the animals treated with IUdR than in the control animals. The clinical importance of this observation remains to be clarified.

Herpes varicella virus is the etiologic agent responsible for varicella (chicken pox) and herpes zoster (shingles). IUdR inhibited markedly the replication of the varicella virus in tissue culture (292), and this finding suggested the systemic use of IUdR in the treatment of patients with varicella-zoster infections, a condition that can be fatal. Rapp and Vanderslice (291) found that IUdR prevented both the spread and the cytopathic effects of zoster virus in human embryonic lung cells *in vitro*, but the infection was not eliminated. The treatment of 52 patients with herpes zoster by topical application of a 0.1% solution of IUdR was not effective (244). It was pointed out that the rash observed in this condition may result from the effect of the virus in the posterior root ganglia and that the mode of topical application may not have resulted in the absorption of the drug to the site of viral replication.

The treatment of herpes genitalis in man has been described in 2 reports and statistically valid beneficial effects were reported (169, 314). Thus, Schofield (314) reported that all 50 of his patients responded well when IUdR in 0.5% ointment was applied 3 times daily for 2 to 7 days, while in 22 patients who had also had previous episodes of herpes the time required for healing of the lesions was reduced to one-half (P .001). In cattle infectious bovine rhinotracheitis virus produces conjunctivitis, vaginitis and balanoposthitis, for which there has been no therapy. IUdR strongly inhibits replication of this virus in cell culture and, when applied to rabbit skin within 1 hour after infection, prevents the development of lesions (269). IUdR inhibited the cytopathic effects produced by a strain of bovine malignant catarrhal fever virus (273). The formation of infectious murine cytomegalo virus was inhibited by IUdR, but the cytopathic effects were not prevented although the inclusions were smaller and less well defined (155).

IUdR, 5-iodo-2′-deoxycytidine and cytosine arabinoside administered into the cerebrospinal fluid of rabbits did not prevent death produced by a neurotropic strain of herpes simplex virus applied to the cornea 3 days earlier (195). Similarly Benda (28) found that in rabbit cornea infected with B-virus (herpes virus simiae), although IUdR delayed the appearance of lesions on the cornea as well as generalized conjuntivitis, there was no effect on the survival time. Force and Stewart (100) reported inhibition by IUdR of the development of encephalitis

due to Columbia-SK virus in mice, the mechanism being prevention of uptake of the virus into the brain.

Since treatment of the various localized infections with IUdR succeeded because one could achieve adequate concentrations in a restricted area, it was important to determine whether systemic viral infections could be suppressed. The experience of Calabresi *et al.* (47) and Welch and Prusoff (361) in the treatment of neoplasms in man by systemic administration of IUdR led to its study in the therapy of smallpox. Calabresi *et al.* (50) showed that vaccinia infections, both in rabbits and in patients with advanced neoplastic disease, could be suppressed by the intravenous administration of well tolerated amounts of IUdR. In patients who were capable of converting 5-iodo-2′-deoxycytidine rapidly into IUdR a similar beneficial effect was observed with the deoxycytidine derivative (48). Although the acute toxicity (stomatitis, hair loss, hematopoietic depression) observed was reversible, these investigators recommended that the use of this compound be restricted to patients with severe or potentially lethal DNA-viral infections, because incorporation of IUdR into the DNA of the genome of the host cell conceivably could express itself by genetic damage, infertility, or even neoplastic change (360). The topical application of IUdR in an ointment to sites of inoculation with vaccinia virus in man was found by Bjornberg and collaborators (33) to produce no significant effect.

Although FUdR, BUdR, and IUdR inhibit antibody formation *in vitro* (79, 80), BUdR but not IUdR inhibited the immune response in a mouse test system (32, 87) and in a rabbit system (49). Fischer *et al.* (98) found cytosine arabinoside to be the most potent inhibitor of the primary immune response in mice; FUdR inhibited in lower doses than BUdR and in practical dosage levels IUdR had little effect (98).

The halogenated derivatives of deoxyuridine (5-iodo-, 5-bromo-, 5-chloro- and 5-fluoro-, *i.e.*, IUdR, BUdR, CUdR, and FUdR) have produced teratogenic disturbances in embryos of rats, chicks and mice (56, 68, 192). The ability of 5-iodouracil and 5-bromouracil to affect the mutation rate in bacteria and the ability of BUdR to produce chromosomal damage are well established. Fahmy *et al.* (92) compared the mutagenic properties of BUdR, IUdR, cytosine arabinoside, FUdR, 6-azauridine and 6-azacytidine in *Drosophila melanogaster* and found their activity to be roughly the same and hence not related to incorporation into DNA. None of the compounds induced the point-mutations looked for, and the only chromosome deletions observed were those small ones that result in phenotypic change. Although point-mutations are observed with some of these compounds in bacteria and bacteriophage, they are not observed in *Drosophila melanogaster*; and the authors concluded that these compounds would not produce long-term genetic hazards in man. Despite impressive evidence for the predictive value of *Drosophila* for mammalian species (92), there are innate hazards in the projection of results from these species directly to man; of pertinence to their study is the question of whether IUdR or BUdR was incorporated into the DNA of *Drosophila* in these experiments. Thus, the precautions expressed earlier with respect to the use of IUdR (360) must be observed until more direct evidence

reassures us as to its safety. The hazards must be regarded as acceptable, however, when IUdR is to be used in the treatment of either potentially lethal neoplastic disease or of potentially lethal infections with certain DNA viruses.

The amount of IUdR administered during treatment of herpes keratitis is about 0.001 of the amount that may give rise to toxicity in man (47, 361). Bakhle and co-workers (16) studied the systemic toxicity that might result from the absorption of IUdR after topical application to the cornea of rabbits. About 0.1 % of the administered dose of radioactive IUdR instilled into the conjunctival sac of rabbits was retained in nonocular tissues, the ovaries containing no IUdR in polymeric form. They concluded that the amount of IUdR used in the treatment of the cornea infected with virus does not constitute a hazard even if all the drug is absorbed systemically. This is related to the rapidity of catabolism of IUdR *in vivo* (361).

Of particular importance to the eventual use of an antiviral agent in the treatment of smallpox in man was the observation that suppression of the virus-induced lesions in rabbits occurred when IUdR was administered 48 hours after infection (50). Comparison of the antiviral activities of IUdR (120 mg/kg), 6-azauridine (300 mg/kg) and methisazone (20 mg/kg) (see Section V) in weanling rabbits bearing a severe systemic vaccinial infection showed that azauridine was moderately effective under conditions in which the other 2 drugs exhibited similar but much greater antiviral activity (93). Rada and Blaskovic (284) found 6-azauridine requires 100 times the concentration of IUdR to inhibit vaccinia virus in HeLa cells.

A preliminary clinical trial of IUdR in the treatment of patients with smallpox was begun recently in Madras by Rao *et al.* (285a). Only 3 patients with early smallpox, of types judged by criteria established by Rao to be highly lethal, have been treated with an intravenous dose of 15 mg of IUdR per kg every 8 hours for 5 days; of these, 2 survived. Significant toxic effects were not observed and further studies are planned for 1967. Although methisazone can prevent smallpox in persons exposed to patients with smallpox, it has no therapeutic value after onset of even the early manifestations of the disease. It is in this latter situation that IUdR could prove to be of value, in view of the results obtained with vaccinial infections.

Breeden *et al.* (39) treated a patient with encephalitis due to herpes simplex virus with an intravenous injection of IUdR (total dose of 39 g) during a 7-day period after surgical decompression, and the patient recovered. Although no firm claim was made for the efficacy of IUdR, further trials of the drug in this condition are indicated.

In cell culture complete eradication of vaccinia virus was not achieved by the use of a single drug, but a combination of BUdR with 5-methyltryptophane, the antibiotic noformicin [N-(2-amidino-ethyl)-5-imino-2-pyrrolidine-carboxamide], or methisazone resulted in complete eradication of vaccinia virus and recovery of normal HeLa cells (110, 111). Cytosine arabinoside, mitomycin C, or both, in combination with noformicin or methisazone was toxic to cell cultures (110, 111).

One of the major problems in the clinical use of IUdR is its extremely rapid metabolic degradation, which undoubtedly limits its incorporation into DNA (361). Attempts have been made to increase the amount of IUdR incorporated into DNA either by the concomitant use of amethopterin, FUdR, or thymine riboside or by varying the vehicle in which IUdR is suspended (see 278). More recently, Langen and Etzold (221) enhanced the incorporation of IUdR into the DNA of tissues in the cat by administering thymine deoxyglucoside, an inhibitor of pyrimidine nucleoside phosphorylase; however, the enzyme present in normal tissues of rodent, horse, and man as well as neoplastic tissue of man is not inhibited by this analog. There are 2 enzymes that cleave pyrimidine deoxyribonucleosides, namely, uridine-deoxyuridine phosphorylase and deoxythymidine phosphorylase; but only the former is inhibited by thymine deoxyglucoside, and it is the only one present in the cat, in contrast to other animal species. A novel approach to this problem was described by Woodman (369), who complexed IUdR or 5-iodo-2′-deoxycytidylic acid to polyethyleneimine, a polycation, with and without menadione sodium diphosphate (Synkavite), and observed a 2- to 3-fold increase in the amount of iodine associated with tumor DNA. Unfortunately, this preliminary report did not provide evidence that the iodine observed in the purified DNA represented incorporation into the DNA-polymer and not merely the formation of a complex between DNA, poly*anion*, and the IUdR-complexed poly*cation*.

B. Mechanism of action

The halogenated deoxyribonucleosides are of interest not only because of the many biochemical reactions that they affect, but also for their application in areas of genetics, cell biology, radiobiology, and neoplasia, as well as in virology. The halogen in the 5-position of the pyrimidine moiety is responsible for the specific physical and biological properties of the halogenated deoxyribonucleoside. A few comments will be made about the chemistry of these compounds in order to illuminate their biological properties.

The size of halogen atoms, as defined by their van der Waals radii, varies from 1.35 Å for fluorine to 2.15 Å for iodine. In systems that can incorporate free pyrimidines into nucleic acid, 5-fluorouracil enters only RNA, while 5-bromouracil and 5-iodouracil enter DNA, and the 5-chloro-derivative (chlorine has a van der Waals radius intermediate between that of fluorine and that of bromine) may enter both RNA and DNA. Although uracil is not found in DNA, this is not because deoxyuridine triphosphate is not a substrate for DNA-polymerase instead of deoxythymidine triphosphate, but because accumulation of deoxyuridine triphosphate cannot occur, as it undergoes rapid enzymic breakdown (31, 131). Okazaki and Kornberg (263) have shown that deoxyuridine triphosphate, and to a lesser extent FUdR triphosphate, can substitute partially for deoxythymidine triphosphate in the reaction catalyzed by DNA polymerase. Thus, the failure of incorporation of FUdR into DNA is related to the failure of incorporation of deoxyuridine.

In halogenated pyrimidines, the electron configuration of the ring is altered because of the inductive effect of the halogen, and this results in more acidic dissociation constants. The pKa of thymidine is 9.8, whereas that of IUdR is 8.25. Since the N-1 position is substituted with the deoxyribose moiety, the proton on the N-3 position must be dissociated. This phenomenon may be crucial to the antiviral activity of IUdR and will be discussed in more detail below. Camerman and Trotter (53) examined the crystal and molecular structure of IUdR by X-ray diffraction and proposed that the antiviral activity may be related to the unusually short intermolecular distance of 2.96 Å between the iodine and the oxygen of a carbonyl group, whereas the sum of their van der Waals radii is 3.55 Å. They postulated that the ability of iodine to form charge transfer bonds may cause the increased interchain attraction that could either prevent or delay the synthesis of virus-DNA, thereby permitting normal host defense mechanism to operate.

The energy of the carbon-halogen bond varies considerably, that of carbon-fluorine being considerably higher than that of either carbon-iodine or carbon-bromine. Both FUdR and IUdR are catabolized to the free halogenated pyrimidines, 5-fluorouracil and 5-iodouracil, respectively; but subsequent degradation results in extensive liberation of iodide, but not of fluoride.

Where the size of the substituent in the 5-position of the pyrimidine is important, FUdR mimicks deoxyuridine, while BUdR and IUdR mimic thymidine. Where bulk in the 5-position of the pyrimidine component of this molecule is not critical, all the halogenated derivatives serve almost equally as substrates for the same enzyme.

5-Fluorouracil, FUdR and 5-trifluorouromethyldeoxyuridine were synthesized by Heidelberger of the University of Wisconsin and have been studied extensively as biochemical tools, as cancer chemotherapeutic agents, and more recently as potential antiviral agents. FUdR and 5-trifluoromethyl deoxyuridine, after phosphorylation, inhibit thymidylate synthetase but by what appears to be different mechanisms. At first, both flourinated nucleotides exhibit competitive kinetics, but after 10 minutes of incubation with the trifluoromethyl analog before adding normal substrate into the reaction mixtures, noncompetitive kinetics is seen. Whereas 5-trifluoromethyl-2′-deoxyuridine may be incorporated into the DNA-polymer, FUdR, as indicated above, does not enter DNA; but it may be incorporated into RNA after cleavage to 5-fluorouracil, with subsequent ribonucleotidation. The greater inductive effect of the increased number of fluorine atoms causes the pKa of 5-trifluoromethyluracil to be 7.35, in comparison to that of 5-fluorouracil, which is 8.15. Whereas the carbon-fluorine bond of 5-fluorouracil is quite stable, that of the 5-trifluoromethyl analog is not; the latter is converted to 5-carboxyuracil, either by mild alkali or metabolically. Once 5-trifluoromethyl-2′-deoxyuridine is incorporated into the DNA polymer, however, it appears to be quite stable to mild alkali. 5-Trifluoromethyl-2′-deoxyuridine has been reported to suppress herpetic keratitis in rabbits (197) and to act against a strain of herpes simplex-virus resistant to IUdR. The tri-

fluoro analog is incorporated into the DNA of bacteriophage T4 (126) and presumably is incorporated similarly into the DNA of animal viruses (references cited in 153).

5-Fluorouracil and FUdR inhibit DNA-viruses (herpes simplex virus, vaccinia virus, SV40 virus, pseudorabies virus, adenovirus and polyoma virus) in cell culture, but have little or no efficacy either *in vivo* (153) or in the agar plaque assay described by Herrmann (157). The difference observed among these methods of assay may be related to the induction of thymidine kinase in virus-infected cells, an event that enables circumvention of the need for thymidylate synthetase, the enzyme inhibited by FUdR monophosphate. In assays of antiviral activity that require diffusion of FUdR either in agar or *in vivo*, there may be a lag in its accumulation into the cells, but sufficient time for the viral induction of thymidine kinase; this enzyme would increase the amount of thymidylic acid, the compound that bypasses the inhibition caused by the monophosphate of FUdR. In cell culture, however, antifolates in the presence of adenine and glycine inhibit the replication of DNA viruses, but not of RNA viruses (155, 232, 308), presumably by blocking the formation of thymidylic acid.

Because of the structural analogy of BUdR and IUdR to thymidine, it is not surprising that these halogenated deoxyribosides and their phosphorylated derivatives participate in reactions that thymidine and its phosphorylated derivatives enter (280). The question that has been posed in an attempt to understand the antiviral activity of IUdR is "are the enzymes concerned with the formation of the viral components, specifically viral-DNA, in the virus-infected cells more sensitive to inhibition by the indicated analog or its phosphorylated derivatives than are the corresponding enzymes of the noninfected cells?" (280). The expectation that a difference might be found in susceptibility to inhibition, and hence in selective toxicity, was based on the many observations of an increase in the activities of enzymes concerned with the formation of precursors of DNA-thymidine, as well as with the polymerization of DNA, which occur upon infection of cells with many of the viruses. The virus-induced enzymes in many instances have physicochemical properties that differ from the corresponding enzyme present in uninfected cells.

In studying the mechanism of the antiviral activity of IUdR, we have tried to identify the enzyme concerned with the formation of virus-DNA that is primarily inhibited, and to find the consequence of incorporation of IUdR into the DNA of the virus.

There are 3 major areas in which BUdR and IUdR or their phosphorylated derivatives are known to exert biochemical effects: (1) competitive inhibition of many of the enzymes concerned with the biosynthesis of DNA-thymine; (2) allosteric or feedback inhibition by IUdR triphosphate, mimicking the normal control activity of deoxythymidine triphosphate; and (3) incorporation into virus-DNA with a subsequent effect on the expression of genetic information during either replication or transcription.

We found no differences in susceptibility to inhibition by IUdR or its monophosphate derivative in the thymidine kinase and thymidylic acid kinase of

mammalian cells before and after infection with herpes simplex virus (280). The triphosphate of IUdR, which was prepared by Dr. P. K. Chang by pyrophosphorylation of the morpholidate of IUdR-monophosphate, competed with thymidine triphosphate in the reaction catalyzed by DNA-polymerase, but again there was no difference between the susceptibilities of the enzymes derived from the infected and uninfected cells (282). In a cell-free system (100,000 × *g*-supernatant fraction) derived from the cells of 2 murine neoplasms (Ehrlich ascites carcinoma and L-5178 leukemia), IUdR triphosphate could replace deoxythymidine triphosphate very efficiently, since the halogenated derivative in the absence of deoxythymidine triphosphate did not inhibit the utilization of radioactive deoxyadenosine triphosphate in the reaction catalyzed by DNA polymerase (281, 282). Okazaki and Kornberg (263) had shown previously, in reactions catalyzed by DNA polymerases derived from *Bacillus subtilus* and *Escherichia coli*, that BUdR triphosphate replaced deoxythymidine triphosphate to the extent of 91 and 93 %, respectively.

The monophosphate of FUdR is a far more potent inhibitor of thymidylate synthetase than the monophosphate of IUdR (15). This is in agreement with the observation by Reyes and Heidelberger (297) that a 5-fold molar excess of deoxythymidylate inhibits the methylation of deoxyuridylate by only 36 %. The inhibition produced by IUdR monophosphate and deoxythymidylate presumably represents those of a pseudoproduct and a product, respectively.

Since thymidine triphosphate not only is the immediate precursor of DNA-thymine, but also is concerned with enzyme control by feedback or allosteric inhibition, which regulates self-synthesis, it was to be anticipated that IUdR triphosphate would mimic deoxythymidine triphosphate in these reactions. Okazaki and Kornberg (263) observed that BUdR-triphosphate is as inhibitory as deoxythymidine triphosphate to thymidine kinase (derived from *E. coli*), under conditions in which neither deoxyuridine triphosphate nor FUdR triphosphate had even marginal activity.

In cell-free extracts derived from 2 murine neoplasms, IUdR triphosphate was about 3 times more inhibitory of thymidine kinase than deoxythymidine triphosphate (281). Deoxycytidylate deaminase, an enzyme believed to be critically involved with the formation of precursors of DNA-thymine, was even more sensitive (by a factor of 10) to the inhibitory effects of IUdR triphosphate than of deoxythymidine triphosphate (281). The triphosphates of deoxyadenosine, deoxyguanosine and deoxyuridine were neither stimulatory nor inhibitory. We confirmed the fact that deoxycytidine triphosphate activated deoxycytidylate deaminase and could not only prevent, but also reverse the inhibition exerted by deoxythymidine triphosphate. The same is true of IUdR triphosphate. Of the enzymes we investigated, deoxycytidylate deaminase is by far the most sensitive to the inhibitory effects of a derivative of IUdR.

In kidney cells of the African green monkey, Diwan and Prusoff (72) could find no difference in susceptibility to inhibition by deoxythymidine triphosphate or IUdR triphosphate of deoxycytidylate deaminase activity before and after infection with herpes simplex virus.

The binding of the deoxyribonucleoside triphosphates by these enzymes is believed to depend upon weak secondary forces, such as hydrogen bonding, salt linkage and van der Waals forces, and to result in conformational changes (allosteric transitions) in the protein. Thus, the inhibition can be removed quickly once the concentration of the inhibitor of the enzymic process is reduced to a low level. The binding of deoxycytidine triphosphate by deoxycytidylate deaminase is either stronger, or at least more influential, than is that of either deoxythymidine triphosphate or IUdR triphosphate, since equimolar amounts of deoxycytidine triphosphate reverse completely the inhibition exerted by preincubation for even 30 minutes with these inhibitors (95a, 281, 282).

Incorporation into DNA is the third area in which IUdR or BUdR could exert a marked effect on the replication of viruses, as well as on that of either mammalian cells or bacteria. The biochemical and physical effects that result from incorporation of halogenated uracils into nucleic acids have been reviewed (41, 88, 207, 280, 340). The physical effects include (1) increased shearing of the DNA polymer during isolation, (2) an increase in the melting temperature of DNA, (3) an increase in the buoyant density, and (4) a small decrease in the pH required for strand separation. Although no effect on transforming DNA has been observed, the biological effects include (1) increased rate of mutation, (2) increased errors in protein formation, (3) inhibition of cellular reproduction, and (4) increased sensitivity to X- and UV-radiations.

Incorporation of halogenated uracil derivatives into the nucleic acids of animal viruses has been demonstrated (81, 189, 279). The incorporation of 5-fluorouracil into RNA viruses can be extensive, and 35 and 50% replacement of the uracil component of poliomyelitis virus and tobacco mosaic virus, respectively, had little effect; but very high levels of 5-fluorouracil did inhibit the formation of tobacco mosaic virus and a 5-fold increase in mutation rate was observed in a virus that had 56% replacement of RNA-uracil by 5-fluorouracil (226). The incorporation of 5-fluorouracil into RNA does produce errors in the formation of proteins (153, 256) as well as abnormal ribosome formation (7, 159, 175). Tershak (348) studied the effect of 5-fluorouracil on the induction of RNA polymerase by polio virus and observed a difference between that formed during growth of polio virus in media supplemented with the analog and that produced when polio virus contained 5-fluorouracil in its RNA. Only the latter produced an enzyme with altered properties even when grown in the absence of drug. These studies confirm the role of the viral genome in directing enzyme synthesis and the effect of incorporation of an abnormal base into nucleic acid. The mechanisms responsible for these abnormalities have been investigated (42, 256). Bujard and Heidelberger (42) investigated in a system composed of a synthetic DNA primer, DNA-dependent RNA polymerase, cytidine triphosphate, tritium-labeled adenosine triphosphate and either uridine triphosphate or 5-fluorouridine triphosphate labeled in the α-position with radioactive phosphorus. On nearest neighbor analysis of the RNA formed, no support was obtained for the incorporation of 5-fluorouracil into RNA in place of cytosine. Whether the cell-free system used is appropriate for such a study or the hypothesis is wrong remains to be ascertained (42).

The DNA viruses, in contrast to RNA viruses, are markedly inhibited when grown in the presence of BUdR or IUdR. These halogenated deoxyribonucleosides have no direct effect on either extracellular vaccinia (279) or herpes simplex viruses (91), and no effect on the adsorption of virus to cells (325), but they inhibit very markedly the formation of infectious virus particles, for the ratio of virus particles to infectious virus may be as high as 10^7:1 (326). The virus particles formed in the presence of IUdR or BUdR are abnormal in appearance (81, 279, 326).

An elegant study of the effects of IUdR and BUdR on the biochemical events involved in the formation of pseudorabies virus has been performed by Kaplan and co-workers (190, 191). These investigators set out to determine whether halogenated virus-DNA was not capable of being encapsulated into a virus particle either for steric reasons or because of an inability to direct the synthesis of functional protein, and they concluded that the latter explanation applied. Virus-DNA accumulated in cells treated with IUdR or BUdR to even a greater extent than in the untreated cells, but IUdR caused an initial lag in the rate of DNA synthesis. Within 16 hours after infection, BUdR decreased the infectious titer by about 2 logs and IUdR by about 4 logs. Whereas the DNA produced in either the BUdR-treated or the control cells was in a form insensitive to deoxyribonuclease, only a very small amount of the DNA formed in the presence of IUdR was insensitive to this enzyme. In contrast to IUdR, BUdR permitted the formation of complete virus particles, even though these were noninfectious.

The DNA that contained IUdR could be incorporated into virus particles provided the infected cells were incubated with thymidine either before or after the formation of this halogenated DNA. These observations support the concept that replacement of DNA-thymine by 5-iodouracil results in the formation of either no protein coat or an inadequate one. The addition of thymidine to the infected cells grown in the presence of IUdR presumably enabled the synthesis of an adequate amount of DNA that permitted proper protein synthesis, with a resultant maturation of virus. The virus particles that contain DNA substituted with BUdR were postulated to be noninfectious because of either an inability to induce early enzymes or a failure to be adsorbed, to penetrate or to uncoat because of a faulty protein coat (188, 190). In contrast, Hirt (161) reported that incorporation of BUdR in 1 or 2 strands of polyoma virus DNA has no adverse effect on replication. This may be related to the amount of incorporation of this analog.

In HeLa cells infected with herpes simplex virus, Schneweis (313) showed that the addition of BUdR markedly inhibited viral replication but did not prevent a strong cytopathic effect. The fact that the virus particles produced in the presence of BUdR appeared to have lost their ability to be adsorbed may account for the loss of infectivity. This result implies that BUdR directed the formation of an inadequate coat protein.

In normal and IUdR-labeled DNA derived from vaccinia virus by a modification of Kleinschmidt's method, McCrea and Lipman (245) found the IUdR-substituted DNA liberated by osmotic shock to be extensively fragmented, in

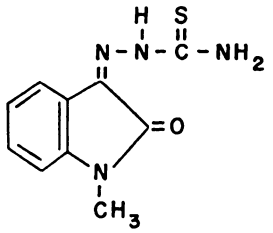
contrast to the normal DNA, which was consistently relatively long and continuous. This increased fragility of the halogenated DNA was accompanied by a minimal alteration in buoyant density, no change in the temperature of melt (Tm), and no increased sensitivity to UV- or γ-radiations. These investigators proposed that the decreased pock-forming ability of the IUdR-substituted virus particles may be attributed, at least in part, to the increased fragility of the DNA when released within the host cell by the "uncoating enzyme." Whether the procedure used for isolation of the halogenated DNA resulted in the increased fragility or whether this condition exists within the intact virus particle must still be elucidated.

5-Bromouracil, 5-iodouracil and their deoxyribonucleosides increase the mutation rates in bacteria and bacteriophage (30, 105, 167, 227, 228, 233). The pKa of thymidine is 9.8, whereas those of BUdR and IUdR are 8.1 and 8.25, respectively; accordingly, at pH 7, 0.16% of thymidine is in the anionic form, in contrast to 8.0% for BUdR. Theoretically, as compared to thymidine, the halogenated deoxyribonucleosides have about a 50-fold greater probability of base-pairing with guanine, either during replication of DNA or during the formation of messenger RNA. This has been confirmed in Kornberg's laboratories (350) in a study of the amount of guanine nucleotides polymerized when polymers composed of adenine and thymine or adenine and 5-bromouracil were used as primers. Whereas the adenine-thymine polymer resulted in an incorporation of less than 1 guanine per 100,000 nucleotides polymerized, that formed by the adenine-5-bromouracil primer contained 1 guanine per 2,000 to 25,000 nucleotides polymerized. Nearest-neighbor analysis of the incorporated guanine did not reveal exclusive incorporation of guanine next to 5-bromouracil, as would have been predicted; and this was attributed to a difference in the secondary structure of the synthetic primer in comparison to that of DNA under conditions *in vivo*, where 5-bromouracil may indeed direct the incorporation of guanine (350).

Lorkiewicz (233) compared the amount of IUdR incorporated into the DNA of *E. coli* with the frequency of mutation to streptomycin resistance; 15 to 20% replacement of DNA-thymine increased frequency 3-fold, and 50% replacement did so 20-fold. Since the alterations induced by 5-bromouracil in T2 and T4 phage differed from those occurring spontaneously, Benzer and Freese (30) regard the effect of 5-bromouracil to be at specific locations of the DNA rather than merely by enhancing by several hundred-fold the rate of spontaneous mutation. The mutagenic effect produced by BUdR in phage S13 was regarded by Howard and Tessman (167) to be the result of transition mutations, the induction of which can be suppressed by thymidine, presumably by competing with BUdR for incorporation into the DNA. It is curious that incorporation of 5-bromouracil or 5-iodouracil into DNA of *Bacillus subtilus* had no marked effect on its specific transforming activity; yet there was a decrease in the transforming activity for certain genetic markers (89, 341).

It is clear that there are numerous biochemical sites at which either IUdR or its phosphorylated derivatives exert inhibitory effects. It is not clear which locus represents the primary site of inhibition. Those enzymes that are inhibited are

not inactivated and they resume their catalytic activities as soon as the concentration of the appropriate derivative of IUdR is sufficiently diminished. IUdR does not appear to inhibit the formation of DNA, but rather permits the synthesis of an abnormal DNA, which contains IUdR in place of an appreciable amount of a normal component, thymidine. It is in events subsequent to incorporation of IUdR into viral DNA that the answer is being sought in regard to its mechanism of action. Is a fraudulent messenger RNA made? Are fraudulent proteins made? What is the nature of the interaction of the messenger-RNA made by transcription of halogenated DNA, with polyribosomes?



N-Methyl isatin β-thiosemicarbazone

V. METHISAZONE AND OTHER ISATIN-β-THIOSEMICARBAZONES

The antiviral activity of several thiosemicarbazones has been established in several laboratories; methisazone (N-methylisatin-β-thiosemicarbazone) is a chemoprophylactic agent in man, useful especially against the smallpox virus (349). Although the N-methyl derivative is not the most active derivative of isatin-β-thiosemicarbazone, it was chosen for extensive study because of economy of production (24). The N-ethyl derivative is about 40% more active than the N-methyl compound (307). Unsubstituted isatin-β-thiosemicarbazone inhibits the reproduction of vaccinia virus in mice (23), but the N-ethyl derivative inhibits it more at the same molar dose (26). N-ethyl isatin-β-thiosemicarbazone has been reported to suppress neurovaccinia infection of mice (274).

The effectiveness of this substance in preventing smallpox in man has been established (27). During a smallpox epidemic in Madras, 2,227 persons who had had close contact with established cases of smallpox were vaccinated and, of these, 1,101 received methisazone. The group that did not receive the drug developed 78 cases of smallpox, of whom 12 died, whereas the group on drug therapy had only 3 mild cases of smallpox and no deaths. These studies have been enlarged and a preliminary report presented by Bauer (24) indicated that among 2,297 treated close contacts, only 6 developed smallpox, of whom 2 died, whereas among 2,842 untreated contacts, 114 developed smallpox and 20 died. The only significant side-effect of the drug was vomiting.

Ribeiro do Valle and co-workers (298) observed a similar efficacy of methisazone among persons exposed to alastrim in Brazil. Forty-two cases of alastrim developed among 520 contacts who received no drug, whereas among 384 treated contacts only 8 developed this disease.

In 10 patients with vaccinia gangrenosa treated with methisazone, 5 recovered, and, of 22 patients with eczema vaccinatum, 12 were affected beneficially (see 24). Hansson *et al.* (144) also found that methisazone stopped the progression of vaccinia gangrenosa. A provocative preliminary report by Sandeman (309) indicates that methisazone may have benefited some patients with malignant lymphoma. This very important observation merits independent confirmation.

Rao and co-workers (286, 287) investigated the efficacy of 3-methyl-4-bromo isothiazole-5-carboxaldehyde thiosemicarbazone (M. and B. 7714) in the prevention of smallpox in man because this compound suppressed neurovaccinia and rabbit pox in animals. The drug had some beneficial effect but the fatality rate among those that developed smallpox was similar. Furthermore, the drug was not well tolerated, the major side effect being vomiting.

The development of resistance to methisazone by rabbit pox virus in mice has been described (5), a finding that bears on the future use of this agent in the treatment of smallpox.

Methisazone inhibits the reproduction not only of pox virus but also of adenovirus types 3, 7, 9, 11, 14, 16, 17, 21 and 28 and of SV15, a simian adenovirus (25). Adenovirus 11 was inhibited even when the drug was added 13 hours after infection. Thus the range of effectiveness of this drug has been extended.

The mechanism of action of the thiosemicarbazone derivatives has been under active investigation. No effect is exerted on adsorption of the virus to cells, its penetration, or the synthesis of viral DNA. Although the synthesis of most of the virus proteins is not affected, the formation of a few of the "late" proteins is (6), and this may be the cause of the formation of what appear to be immature vaccinia particles that are noninfectious. Intercalation into DNA has been considered as a possible mechanism of inhibition (306); Magee and Bach (238), however, detected an interaction of DNA with only very high concentrations of the drug, and they found no effect of isatin thiosemicarbazones on the induction of thymidine kinase or DNA polymerase or on the formation of viral DNA. Removal of the drug permitted a portion of the formed DNA to be incorporated into infectious particles. Whereas dactinomycin prevented the normal "shut off" of synthesis of thymidine kinase (242), the isatin derivative had no such effect.

The interesting finding by Appleyard *et al.* (4) that dactinomycin prevented the inhibition by isatin-β-thiosemicarbazone of virus replication at concentrations of the former that did not interfere with virus reproduction suggested to them that the drug induced the formation of a specified RNA that directed the synthesis of a protein that was the true inhibitor. Woodson and Joklik (370) found that m-RNA synthesized about 3 hours after infection with vaccinia virus could not be properly translated and its sedimentation coefficient had decreased from 16S to 8S. The early viral m-RNA was not affected, as evidenced by the normal appearance of the "early" enzymes, as well as some structural virus proteins. Thus, the replication of virus-DNA, synthesis of viral m-RNA and the functioning of "early" viral m-RNA are not affected by this drug.

Joklik (180) reported that methisazone destroys polyribosomes, thereby preventing attachment of m-RNA, which subsequently is degraded. Presumably

this time sequence permits the synthesis of the "early" proteins, but would prevent the synthesis of "late" proteins that may be critically involved in maturation or assembly of virus particles.

Thus methisazone has proved to prevent smallpox in man, when the drug is used during the incubation period of the disease, but not subsequently. Its mechanism of action is still under investigation.

VI. INTERFERONS AND ENHANCERS

Interferon was discussed in 2 recent reviews (20, 96). Interferons are believed to play a role in the process of recovery from viral infections before antibodies develop. The term is applied to a class of basic polypeptides of molecular weights ranging from 25,000 to 31,000; in addition, the name "interferons" is used for materials that are not induced by viruses; these have molecular weights that are higher, about 87,000. Interferons are produced by the host cell in response to the virus particle, to virus nucleic acid or to nonviral agents. They are not present in the uninfected cell in detectable amounts, but after exposure to the appropriate stimulus, their production is directed by new m-RNA transcribed from the host cell DNA, a process that is inhibited by dactinomycin (357). Once m-RNA has been formed, dactinomycin does not inhibit the synthesis of interferons. By interfering with cellular RNA synthesis, virulent myxovirus can prevent the synthesis of interferon induced by myxovirus made avirulent by UV-irradiation (357). If the host cell is irradiated with UV before infection with virus, the yield of interferon is decreased and the size of plaques formed is increased (60). Human interferon was prepared by Merigan and co-workers (248) by infection of human neonate foreskin fibroblasts with Newcastle disease virus. It has a molecular weight of 26,000, is stable between pH 2 to 10, susceptible to trypsin digestion, and relatively stable to heat, and has an isoelectric point near neutrality (248). Interferon of molecular weight more than 100,000, present in rabbit serum, was separated by Sephadex chromatography into 2 peaks of activity having molecular weights of 40,000 and 56,000, respectively (201).

In cells of a particular species unrelated viruses may induce the formation of interferons that appear to be identical biologically and physically; but in cells of different animal species an individual virus induces interferons that are physically different. Although interferons lack virus specificity, there appears to be strong but not necessarily absolute cell specificity. Thus, the problem of production of adequate amount for clinical investigation might be bypassed by stimulation of the host animal to synthesize his own interferon.

Statolon, an anionic polysaccharide produced by the mold *Penicillium stoloniferum*, when added to cell culture causes the formation of a substance that has all the biological and physical properties of virus-induced interferon (215, 250). The injection of statolon, phytohemagglutinin (363), certain bacteria, or bacterial endotoxin (336) into mice produces an inhibitor similar to interferon; this appears in peak levels within 2 hours, whereas virus-induced interferon requires 6 to 12 hours. Helenine, a substance derived from *Penicillium funicolosum*, induces the appearance of "interferon" at a rate similar to that of viral induction

(303). The "early interferon" may be present normally and is released by those cells with which endotoxin reacts. Whereas dactinomycin or cycloheximide inhibits the appearance of virus-induced interferon, they do not prevent the release of interferon by endotoxin (140). Interferon induced by statolon or endotoxin *in vivo* has a molecular weight of about 89,000.

Youngner and Stinebring (372) found that a second dose of either endotoxin or statolon given to mice intravenously after 48 hours released less interferon than the first dose. However, treatment of mice with either agent did not affect the response of the other. Both agents increased the levels of interferon in mice previously infected with virus. These investigators concluded that although the interferons released by endotoxin and statolon are of similar molecular weights (about 90,000), they are released either from different cells or by different mechanisms. The kinetics and site of interferon production in rats were investigated by Rossum and Somer (301), who found that interferon appeared in the serum within a few hours and that the spleen had the highest titers. This has been attributed to the high concentration of reticuloendothelial cells, which remove the circulating virions. Lung, kidney, liver and serum had titers that were about 5% that of the spleen. The question still remains whether interferon is produced primarily in the spleen or whether interferon accumulates in this organ because of the high concentration of virus there. It would be of interest to explore the synthesizing capacity of these organs in an *in vitro* system.

A TRIC agent (LB-1), a member of the psittacosis-LGV-trachoma group, has been reported by Hanna and co-workers (142) and Merigan and Hanna (249) to induce an interferon with a molecular weight of about 50,000, both *in vivo* and *in vitro*. The peak of this interferon production in the serum of mice, like that induced by a virus, is reached by 6 to 13 hours. Virus-induced interferons inhibit the replication of the TRIC agent.

Whether the synthesis of DNA is required for the formation of interferon has been investigated. Holmes and colleagues (164) found that IUdR in relatively high concentrations (3×10^{-4} M) inhibited the production of interferon induced by mumps virus, whereas Burke and Morrison (43) observed that the amount of either IUdR or aminopterin required to inhibit DNA synthesis in chick embryo cells had no effect on interferon production, although 100-fold increase in IUdR (2×10^{-3} M) inhibited the cellular synthesis of RNA and depressed the production of interferon. Donikian and Stewart (73) found that IUdR either had no effect or increased the production of interferon by chick embryo cells infected with irradiated influenza virus. Similarly, FUdR, in concentrations that inhibited total DNA synthesis and reproduction of vaccinia virus by 95%, had no effect on the production of interferon by chick embryo cells challenged with either an RNA or a DNA virus (225). However, Coto and co-workers (65) observed inhibition by FUdR of the production of interferon in chick embryo cells inoculated with influenza virus that had been irradiated with UV. Although it appears that DNA synthesis is probably not required for the formation of interferon, controversy still exists.

That the host cell genome is the basis of interferon synthesis induced by virus

is supported by the observation that irradiation of rat embryo cells before infection decreases the yield of interferon (60). The primary lethal effect of ultraviolet radiation is exerted on DNA with the formation of various pyrimidine dimers and hydrates.

A disturbing development is the finding that the cell also can produce substances that counteract the effectiveness of interferon and thereby enhance viral replication. These substances have been termed "enhancer agents" or "stimulon." The action of "enhancer" is blocked by interferon and the effectiveness of interferon is decreased by "enhancer" (193). A substance termed "blocker," found in impure preparations of interferon and allantoic fluid of eggs, has been partially characterized and resembles interferon in many physical properties (172). This substance interferes with the formation of interferon, and since "blocker" is more readily apparent after infection of chick embryo with virulent viruses its possible role in virus virulence was considered. Certain polio viruses as well as extracts from cells infected with these viruses were observed by Ghendon and co-workers (118) to antagonize the antiviral effect of interferon. This substance was termed "anti-interferon" and is assumed to be a protein coded by the virus genome.

The mechanism of action of interferon as an inhibitor of both RNA- and DNA-viruses, as well as of viral induction of enzymes, is unknown. Interferon has no effect on extracellular virus or on its adsorption, penetration or release. Although interferon inhibited the induction of thymidine kinase in vaccinia-infected cells (261), the inhibition of vaccinia virus replication was greater than the suppression of the production of thymidine kinase (119); it is possible that other essential enzymes may be affected more markedly by interferons. The fact that dactinomycin, *p*-fluorophenylalanine and puromycin inhibit the antiviral activity of interferon suggest that protein synthesis is necessary for its antiviral activity. It has been proposed that interferon induces synthesis of a new m-RNA that codes for a cellular protein and that this is the true antiviral substance in cells (106, 107, 223, 229, 344).

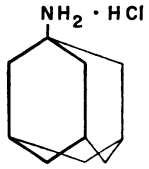
Although no pure interferon has been isolated as yet, the available findings provide a basis for the species specificity of interferon. Dactinomycin does not prevent the formation of interferon late in viral infection presumably because a stable m-RNA has been formed already, whereas the inhibitors of protein synthesis that function after m-RNA formation do inhibit at all times the normal synthesis of interferon. The protein synthesized by interferon has been postulated to be a nuclease capable of degrading not only the viral nucleic acid but also cellular RNA (329).

The best therapeutic results are obtained when interferon is administered before viral infection but antiviral activity is exerted also when the material is given within a few hours, but not more than 24 hours, after infection; it appears to be of little value in a well established viral infection. Antiviral activity has been demonstrated in man when interferon is given before the virus. In animals, interferon administered intravenously is rapidly cleared from the blood, 86% in 30 minutes and 97% in 1 hour; about 17% of the material appeared in lungs and kidneys (339). Interferon prepared in monkeys and applied to the eyes of man

infected with vaccinia virus led to quicker healing of the superficial, but not the deep stromal infection (183). When interferon was given intradermally to man 24 hours before smallpox vaccine, the response to the vaccination was either prevented or depressed (171). Endogenous interferon induced by a measles vaccine prevented the otherwise successful vaccination against smallpox; this action suggests the possibility that generalized vaccinia and vaccinia gangrenosa may be caused by a lack of formation or release of interferon either locally or systemically (270).

It is clear that the predominant action of interferon is a prophylactic one and that the material has only limited use after virus replication has been initiated. Of possible clinical interest is the observation by Levy and co-workers (226) that the previous administration of interferon caused new interferon to appear earlier after infection of chick embryo cells with virus and in larger amounts than in those cells not primed with interferon.

The dose required for clinical trial has been estimated to be 40 mg, an amount optimistically derived, assuming a 100% yield, from 20 liters of fluid present in 2,000 embryonated hen eggs (160). It remains to be seen whether enough endogenous interferon can be induced by substances such as statolon to prevent the spread of a virus infection and thereby permit other host defense mechanisms to abort the infection.



1-ADAMANTANAMINE HCl

VII. AMANTADINE

Amantadine (adamantanamine, Symmetrel) has been under investigation for the treatment of influenza in man and has been approved by the Food and Drug Administration for use in the prevention of illness caused by influenza A2 virus. This synthetic compound does not prevent the adsorption of the virus, but inhibits its penetration into the cell (331). This action would account for the failure of appearance of virus-induced enzymes when cells are infected with virus in the presence of this compound. The antiviral activity of amantadine has been established in tissue culture, chick embryos and mice (70, 133, 259). It is more effective when administered before the virus (influenza, types A, A1 and A2, and parainfluenza 1), as evidenced by the increased survival time of mice. The inhibition of rubella virus in tissue culture by amantadine suggested the use of this compound in the chemotherapy of rubella *in vivo* (235), but studies by Stephenson *et al.* (333) showed its failure in large amounts to prevent or modify the infection of rubella virus in rhesus monkey. The range of effectiveness of this compound has been extended by Wallbank and co-workers (358) by the demonstration of the inhibition of 2 oncogenic viruses, Rous and ESH sarcoma viruses.

In agreement with what has been observed with other viruses, it inhibited cell penetration by the viruses.

In an Asian influenza epidemic Wendel *et al.* (362) found that the administration of amantadine before detectable infection resulted in illness of 5 of 439 (1.1 %) treated persons and 15 of 355 (4.2 %) in the placebo group. There was no effect in persons already exhibiting the disease. A shortcoming of the study was that it was not established that the influenza virus was the etiological agent (362). Patients with established upper respiratory tract infections showed no significant difference from the placebo group in response to amantadine. Although these persons had symptoms characteristic of influenza, it was not established in each case that the influenza virus was the etiological agent. The effect of amantadine and a placebo on the serological response in man produced by an attenuated influenza virus was studied by Jackson and colleagues (176) and those subjects treated prophylactically with amantadine formed less serum antibody. When treatment was given simultaneously with the infection or 3 hours later, no significant reduction in infection was apparent (332). A disturbing note is the finding of Cochran and co-workers (59) that treatment with amantadine aggravated influenza in ferrets.

Amantadine is recommended by the manufacturers for prophylaxis in persons with cardiovascular, pulmonary, renal and metabolic disorders in whom illness due to the influenza A2 virus would compound the already existing debilitating disease. This drug should be of value during an epidemic caused by this specific virus, but no prevention of illness due to any virus other than influenza A2 has been shown. The recommended adult daily dose is 200 mg, and although it is stated that oral doses of 300 mg and 400 mg per day may cause marked central nervous system disturbances, these may be seen in some patients at the recommended dose of 200 mg per day. It is to be hoped that this drug will be the forerunner of a drug that has a broader spectrum of activity among the influenza viruses and preferably a greater therapeutic index.

VIII. PYRIMIDINE ARABINOSIDES

Various purine and pyrimidine β-D-arabinofuranosides have been synthesized and found to possess marked antiviral activity. Several reviews have appeared recently describing the biochemical and biological properties of the D-arabinosylnucleosides (62, 302, 324). The arabinosides of cytosine (cytosine arabinoside or cytarabine), thymine, 5-bromouracil, 5-iodouracil, and 5-chlorouracil inhibit the reproduction of DNA viruses, for example, vaccinia, herpes and pseudorabies (10, 112, 295). Whereas the inhibitory effects of the arabinosides of thymine and the halogenated uracils are reversed by thymidine, that of cytosine arabinoside is reversed only by deoxycytidine.

RNA viruses in general appear to be resistant to the inhibitory effect of cytosine arabinoside (45, 296, 351), but Rous sarcoma virus, an RNA virus, is sensitive to it (85). Although cytosine arabinoside is as effective as IUdR in inhibiting the replication of herpes simplex or vaccinia virus (296), cytosine arabinoside is significantly more cytotoxic (196, 354). This is unfortunate since strains of herpes

simplex virus resistant to IUdR are sensitive to the inhibitory effects of cytosine arabinoside (45, 396, 354). Development of appreciable resistance to cytosine arabinoside by viruses has not been observed (295, 354, 355).

The primary site of inhibition of cytosine arabinoside has not been elucidated, but as with IUdR (283) several potential biochemical areas have been implicated. Chu and Fischer (57) have presented evidence compatible with an inhibition of the reduction of cytidine diphosphate to deoxycytidine diphosphate, and also they and Silagi have demonstrated the incorporation of cytosine arabinoside into both RNA and DNA of L5178Y mouse leukemic cells (58, 321). Whereas neither cytosine arabinoside nor IUdR inhibits the formation of tumor antigen in monkey kidney cells infected with SV40 viruses, cytosine arabinoside but not IUdR decreases the formation of virus antigen (247, 288, 290).

IX. OTHER POTENTIALLY ANTIVIRUS AGENTS

5-Methylamino-2'-deoxyuridine, was synthesized by Visser and co-workers (356) and shown to inhibit bacterial replication (187, 356). Nemes and Hilleman (258) observed this compound to inhibit the replication of herpes simplex *in vitro,* and to suppress experimental keratitis in the rabbit eye. The therapeutic effects in rabbits were equal to those of IUdR or slightly less. This compound shows remarkable specificity for herpes virus, since it exhibits *no* inhibitory activity for other DNA viruses, such as vaccinia and adenovirus type 2, or RNA-viruses, such as Coxsackie B2 and parainfluenza (319). The reason for this unique selectivity, as well as the mechanism of action is unknown.

6-Azathymidine, the 2'-deoxyribonucleoside of azathymine [6-methyl-*as*-triazine-3,5 (2H, 4H)-dione], has been prepared enzymically (277) as well as by chemical synthesis (139), and its biological properties have been reviewed (323). This analog of thymidine inhibits the reproduction of herpes simplex virus *in vitro,* and this inhibition can be prevented completely by thymidine (316). Although 6-azathymidine appeared to affect the formation of the infective virus particle in the same phase of the viral growth cycle as IUdR does, it is much less potent than IUdR (316).

6-Uracil methylsulfone was synthesized by Greenbaum (130) and shown to inhibit the reproduction of several bacteria (165) and of murine lymphomas (138, 177). This compound inhibits the formation of infective herpes simplex virus *in vitro* by a mechanism other than direct inactivation (316). Unsuccessful attempts were made either to reverse or to prevent its inhibitory effect with various levels of thymidine, uridine, or orotate or combinations of these and other precursors of nucleic acids (316).

Hydroxyurea has been reported to inhibit the formation of mature infectious vaccinia virus or T4 phage by preventing the replication of DNA, but not of the formation or function of messenger-RNA of specific enzymes (300). The mechanism of the reversible inhibition of DNA synthesis by hydroxyurea has not been elucidated (299).

Phagacin, a substance found in a soluble fraction of *E. coli* infected with λ-phage inhibits herpes simplex and vaccinia viruses in cell culture, but it is less effec-

tive than IUdR in the treatment of corneal ulcers induced in rabbits by herpes simplex virus. The substance neither produces interferon nor prevents absorption of the virus, but is believed to inhibit its intracellular reproduction, by an unknown mechanism (54).

Congo red inhibits RNA-polymerase (217) and, although it has not been studied as an antiviral agent, it could be of interest since RNA-viruses induce the formation of this enzyme and its continued synthesis is essential for viral-RNA replication. Caliguiri *et al.* (52) found that *guanidine* and *2-(α-hydroxybenzyl)-benzimidazole* prevented the appearance of viral RNA-polymerase, but did not inhibit its activity once it had been formed. Philipson and co-workers (271) reported that the inhibition by guanidine in low concentrations of picorna viruses occurs at an unknown site prior to the synthesis of virus specific RNA-polymerase. It is not clear why guanidine inhibits sensitive strains of polio virus but is required for drug-dependent strains (230). Various amino acids and choline can interfere with the action of guanidine (230, 271).

A great number of substances have been investigated for antiviral activity and a representative few are listed that were reported in 1966 alone: mithramycin (327), ammonium picrate (275), 2-diethylaminoethyl-4-methyl piperazine-1-carboxylic acid (99), substituted morpholinum quaternary salts (3), 1,3-bis(2-chloroethyl)-1-nitrosourea (320), salicylates (170), virothricin (219), N^6(2-hydroxyethyl)adenine (353), extracts of 2 plants (*Magnolia kobus* DC and *Narcissus tazetta* L.) (109), hydroxylamine (94), complex inorganic compounds (206), 2(α-hydroxybenzyl)-benzimidazole (17), cyanate and labile carbamyl compounds (132), glutaraldehyde (36), paolin II from oysters (276), extract of *Mercinaria mercinaria* (184), N′-furfurylbiguanide hydrochloride (312), polyvinylpyrrolidine (251), 3-β-D-ribofuranosyladenine (3-isoadenosine) (116), alanosine (255), phenethyl alcohol (38), D-penicillamine (117), mitomycin C (257), 2,4-dioxo-5-thiazolidine acetic acid derivatives (310, 311).

X. RESISTANCE TO ANTIVIRAL AGENTS, AND ENHANCEMENT OF VIRAL GROWTH

The development of resistance to antiviral agents has been observed both experimentally and clinically. It occurs with both the RNA- and the DNA-viruses. Renis and Buthala (295) have reviewed recently the problem of resistance of viruses to several of the antiviral drugs.

Resistance to IUdR appears to be present naturally to the extent of 0.5 to 4% of the herpes simplex virus population, and the resistant population may be increased to more than 40% by serial passage (325, 326). Herpes simplex virus resistant to IUdR has been selected *in vitro* (45, 295, 352, 354, 355), in rabbit eyes (216, 354), and from patients (198, 220). Vaccinia virus resistant to IUdR has been observed (231) but in rabbit skin the resistant strain was much less pathogenic than the parent population of virus (95).

Dubbs and Kit (74, 75, 208) have isolated strains of herpes simplex and vaccinia viruses that are resistant to IUdR because they are deficient in the ability to induce the formation of thymidine kinase and hence cannot phosphorylate

IUdR. Cells that were not themselves deficient in this enzyme, however, phosphorylated IUdR and therefore inhibited DNA-viral replication upon infection. Humphry and Hsu (168) observed that either high doses of BUdR or low doses of IUdR resulted in the selection of mammalian cells which had low pyrimidine deoxyribonucleoside kinase activity and therefore were resistant to the halogenated deoxyribonucleosides. Cells resistant to IUdR were not cross resistant to cytosine arabinoside or 5-trifluoromethyldeoxyuridine (45, 197, 351, 355).

A strain of virus resistant to IUdR because of a mechanism other than loss of thymidine kinase has been described by Centifanto and Kaufman (55). Several possible biochemical alterations compatible with their observation are subject to direct experimentation. There could be a decreased sensitivity to inhibition of the enzymes concerned with the biosynthesis and utilization of phosphorylated derivatives of thymidine, that is, thymidylate kinase, thymidine diphosphokinase, and DNA-polymerase. There could be larger pools of the various phosphorylated derivatives of thymidine, a situation that would require increased amounts of the analog to exert an inhibitory effect. There could be an increase in the amount of enzymes that are concerned with either the formation of thymidylic acid (thymidylate synthetase) or its subsequent phosphorylation. These possibilities as well as numerous others could account for resistance to IUdR.

Stevens and Groman (334, 335) observed an increased production of infectious bovine rhinotracheitis virus when this herpes virus was grown in the presence of BUdR or IUdR. On subculture of virus derived from cultures treated with BUdR or IUdR, the number of plaques formed increased proportionally to the time of treatment. Pretreatment for 36 hours with BUdR produced an 80-fold increase in plaque count, when compared to those cells that were not treated with BUdR. BUdR-"dependent" virus grew best in cells pretreated with halogenated deoxyribonucleosides (BUdR > IUdR $\gg$ CUdR > FUdR = none), and IUdR-"dependent" virus grew best in cells pretreated with IUdR. There was no absolute dependence on the presence of BUdR or IUdR, but rather a marked enhancement of plaque formation. The mechanism may be related to a possible increase in the size of the cell produced by the analogs, an event known to increase the yield of virus.

Enterovirus resistant to inhibition by guanidine (246) and to hydroxybenzyl benzimidazole have been observed (82). These 2 compounds inhibit the formation of virus RNA polymerase in the sensitive strains, but not in the resistant strains of virus (18, 83).

Recently Appleyard and Way (5) observed the development by rabbit pox virus of resistance to isatinthiosemicarbazone in cell culture and to Methisazone in mice. They expressed concern about the possible development of similar resistance to this compound by the smallpox virus.

XI. CONCLUSION

Within recent years 2 compounds, idoxuridine (IUdR, 5-iodo-2′-deoxyuridine) and amantadine (Symmetrel), have been approved by the Food and Drug Administration for use in the treatment or prevention of disease caused by viruses

in man. The explosive burst of interest in the biochemistry of the reproduction of both RNA and DNA viruses should provide the medicinal chemist with directions for the design of further compounds that have antiviral activity with favorable therapeutic indices. This optimistic hope should be realized within the next decade. The possible if not probable role of viruses in the induction of at least some neoplasms in man is an extremely important direction of present day activities in virology. The prevention of viral infections by vaccines offers great promise of being extended into those diseases at present not under control. However, even if prevention were achieved it would be advantageous to have drugs that could abort those viral infections that do develop. Although these conclusions may appear enthusiastic, they seem justified in view of the rapid developments being made in our understanding of the biochemistry of viruses and their interrelationship with the host cell on a molecular level.

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