POSSIBLE EFFECTS OF ANTIMETABOLITES ON VIRUS REPLICATION

by

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Abstract

Because of the varied opinions and findings concerning the effect of cellular antimetabolites on the replication of both RNA and DNA-containing animal viruses, this project was undertaken to observe the effects of antimetabolites on Newcastle disease and vaccinia viruses: an RNA and a DNA virus respectively. The compounds chosen were the pyrimidine analogues 5-fluorouracil and 5-iodo-deoxyuridine, and thioguanine, a purine antimetabolite. These compounds were chosen because they have already been shown to have growth inhibiting properties in other systems. Inhibition of virus replication was attempted rather than protection of cells, although some effort was made at the outset to determine the tolerance of the 10 - 12 day old chick embryo for the antimetabolites used in this study.

The tolerance of the 10 - 12 day old chick was determined by inoculating the yolk sac of the developing embryo with the analogues and noting the survival time. The maximal concentration of the analogue which allowed 3 out of 4 embryos to survive for 48 hours at 37°C was the dose employed in the tests.

Inoculation of the embryos with the virus was via the allantoic cavity or the chorio-allantoic membrane.

Administration of the antimetabolite was made via the yolk sac either immediately after virus inoculation or 1½ hours later.

After incubation for 36 - 48 hours at 37°C, the allantoic fluid and homogenized chorio-allantoic membranes were assayed to determine whether there was any decrease in the production of virus-specific material in the presence of the antimetabolites.

Tissue cultures of chick fibroblast cells infected with vaccinia virus were treated with antimetabolite and observed for noticeable changes.

Nucleic acid analogues appear to vary in their inhibitory effects on different viruses. The enzymatic mechanism of the inhibitions seems to differ in different organisms. Antimetabolites may inhibit replication of DNA viruses and not of RNA viruses, or they may vary in degree and manner of inhibition among the DNA or RNA containing viruses themselves.

5-Fluorouracil[1] was found to inhibit partially the replication of vaccinia virus in the chick embryo. The antimetabolite appeared to have no significant effect on the replication of Newcastle Disease virus.

There were no observable differences in the titers of either virus harvested from thioguanine treated embryos as compared with untreated embryos.

The pyrimidine analogue, 5-iodo-2'-deoxyuridine appeared to exert some inhibitory effect on vaccinia virus in tissue culture.

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Dr.J.E.Bismanis

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Part I

Studies on Newcastle Disease Virus and Vaccinia Virus in the Chick Embryo

Introduction

The study of chemotherapy in the treatment of viral diseases was developed because of the possible role of numerous compounds in interfering with special metabolic pathways. It is essential that compounds directed specifically against virus-induced metabolic events do not damage normal host cells, or do so to a lesser degree. This is the basis of chemotherapy as proposed by Ehrlich: to cure infectious diseases by chemical agents without injury to the organism affected, i.e., the action of a chemotherapeutic agent must be selective.

Viral diseases in animals pose a major problem for chemotherapeutic studies. Such investigations are of paramount importance since viruses are intracellular parasites and their replication is closely dependent on the metabolism of the host cells. Because of this intimate relationship between virus and host, the use of antimetabolites as chemotherapeutic agents against viruses is limited. Nevertheless, analogues are helpful tools in providing insight into the specific aspects of host cell metabolism and viral synthesis. (Todd, 1959)

Although antimetabolites had been employed in the study of viral inhibition as early as 1947 (Thompson, 1947), it was not until 1962 that a therapeutically useful antiviral agent was found (Kaufman et al., 1962 a, b; Kaufman,

1963). This discovery opened up new avenues in the study of the chemotherapy of viral infections. Purine and pyrimidine analogues have been studied intensively, since the genetic properties of viruses resides in their nucleic acids, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA), which contain the purine and pyrimidine bases in the form of nucleotides.

There is an abundance of informative literature on the effects of various compounds on virus replication both in vitro and in vivo. As early as 1947, Thompson investigated the effect of sodium acetate and sodium malonate on the growth of vaccinia virus, a DNA virus (Thompson, 1947). He observed that these compounds prevented multiplication of vaccinia virus in tissue cultures of chick embryonic tissue, and he deduced that the action probably resulted from the compounds combining with vital thiol groups in the tissue enzyme systems and rendering them unavailable for viral proliferation.

This same worker and his associates studied the effects of antagonists such as 5-bromouracil, 5-nitrouracil and 2,6-diaminopurine on the multiplication of vaccinia virus in tissue culture. 5-Bromouracil and 5-nitrouracil gave small but significant inhibitions, while 2,6-diaminopurine was found to exhibit strong inhibitory effects which were reversed

by purines and nucleic acid derivatives (Thompson et al., 1949, 1950).

Work on the comparative virucidal activities of anti-infective agents on two unrelated viruses was done by Groupé and coworkers (Groupé et al., 1955). They studied influenza A and vaccinia viruses under the same experimental conditions. The biological dissimilarity between the two viruses, an RNA virus on the one hand and a DNA virus on the other, was examined. These two workers reported that the antibiotic actinomycin D at a concentration of 40 micrograms per milliliter (ug./ml.) had no effect on influenza virus at 25°C, and had only a weak effect on vaccinia virus at 37°C when the viruses were cultivated in the chick embryo.

Among some of the pioneer investigations on the effects of fluorinated pyrimidines on nucleic acid metabolism was that of Danneberg. 5-Fluorouridine (FUDR) was found to inhibit cell and virus growth in mice with Ehrlich ascites tumour by preventing synthesis of thymidylic acid and consequently that of DNA (Danneberg et al., 1958). Karnofsky investigated the toxicologic effects of 5-fluoro-substituted pyrimidines in the chick embryo and pregnant rat (Karnofsky et al., 1958, 1960). These experiments provided some of the preliminary information and probably served as an impetus for the use of these antimetabolites.

Major contributions in the area of selective virus inhibition were made by Tamm (1960). He maintained that protection of cells was a more important index of chemotherapy than was mere reduction of virus yield. It was also the more difficult criterion to achieve in successful virus inhibition. Up to this time no agents were known which were useful in the therapy of viral diseases.

By 1961 (Kaplan and Ben-Porat, 1961) investigations began on the mechanism of action of antimetabolites. These investigators proposed that 5-fluorouracil (5-FU) inhibits the synthesis of DNA in rabbit kidney cells in at least two ways: (1) by preventing the formation of thymidylic acid, an essential precursor in DNA synthesis; (2) 5-FU-containing RNA controls the formation of false DNA which is then unable to replicate itself.

Another group of investigators (Reich et al., 1961) obtained significant information on the effect of the antibiotic actinomycin D on the synthesis of cellular nucleic acid and on virus production. Actinomycin D inhibits mammalian cellular RNA biosynthesis, and suppresses the replication of vaccinia virus, a DNA-containing virus. However, cellular DNA synthesis is not affected, nor is the replication of Mengo virus, an RNA virus. From these observations it can be inferred that the replication of viral RNA is distinct from RNA synthesis which is controlled by cellular or viral DNA.

Further studies by these workers showed that the ability of some RNA viruses to infect cells and replicate within them is not inhibited with actinomycin D treatment. They demonstrated that more than 90 per cent of DNA-primed RNA synthesis can be inhibited by actinomycin D in L cells, without significant interference with the production of infectious RNA virus (Reich et al., 1962). Actinomycin acts by complexing with DNA and inhibiting RNA-polymerase synthetic reactions (Goldberg, Reich et al., 1962).

In studies of adenovirus multiplication and cell proliferation, it was proposed that the biological effect of 5-halogenated pyrimidines was most probably due to their interference with the natural bases of nucleic acids (Kjellen, 1962).

The major breakthrough came in 1962 when the pyrimidine antimetabolite, 5-iodo-2'-deoxyuridine (IUDR), was found to be therapeutically active as ananti-viral agent. It was shown to improve or cure rabbit corneal infection caused by herpes simplex and vaccinia viruses, both DNA-containing viruses (Kaufman, 1963), (Kaufman et al., 1962 a, b.) This effect was obtained even after the infection was well established and even when the disease was severe (Kaufman, 1963; Kaufman and Maloney, 1963).

Clinical trials of IUDR were undertaken by Calabresi and his associates (Calabresi et al., 1962; Calabresi, 1963), but there was difficulty in its synthesis and it was very rapidly degraded in vivo. This compound was not originally used in the study of viral disease, but was chosen by these researchers as the most likely agent to be useful in the therapy of disease caused by DNA viruses because of its particular metabolic site of action. It is believed to inhibit the phosphorylation of thymidine and the polymerization of thymidylic acid into DNA. The action on the polymerase system is no doubt somewhat specific, since it is inhibitory in some types of cells, but not in others (Delamore and Prusoff, 1962).

The ability of IUDR to inhibit DNA synthesis and thus the growth of vaccinia virus in vivo was demonstrated by Calabresi (Calabresi et al., 1962). IUDR is an analogue of the pyrimidine base thymidine. The degree of suppression of the response of the virus in this experiment was greater with increasing doses of IUDR, and furthermore, there was no evidence of drug toxicity. These workers inoculated rabbits intradermally with varying dilutions of vaccinia virus cultivated in chick embryo tissue culture.

Studies have also been carried out on the in vitro effects of this pyrimidine analogue in tissue culture (Kaufman and Maloney, 1963; Loddo et al., 1963). The latter group

established that IUDR and FUDR inhibit herpes simplex virus growth both in vivo and in vitro. Vaccinia virus replication in HeLa cells is inhibited by IUDR, but the analogue has no inhibitory activity against polio Type 1 and Coxsackie B_3 viruses, these latter two being RNA-containing viruses.

The formation of vaccinia virus protein in the presence of FUDR was observed by Shatkin, who noted that the rate of viral protein synthesis in HeLa cells in the presence of FUDR was maximal for the first 4-6 hours after infection and continued at a diminished rate during the latter 8-10 hours of the infectious cycle (Shatkin, 1963).

In 1959, Fairman reported that he observed no inhibition of virus production by 5-FU in cells infected with Rous Sarcoma virus (RSV), an RNA-containing virus (Fairman, 1959). On the contrary, Goldé and Vigier (1963) report that incubation of chick embryo fibroblasts infected with RSV in the presence of 5-FU immediately after infection, inhibited both cell growth and virus production. The concentrations of 5-FU required to supress the growth of infected cells were lower than for non-infected controls. Virus production was inhibited only when 5-FU was added to the medium before a certain time. There was no inhibition if 5-FU was added later than 3 days after infection. These authors postulated that inhibition of virus production by 5-FU followed the replccement of

of the pyrimidine base uracil by the antimetabolite in non-viral RNA rather than in viral RNA.

In Goldé's experiment inhibition of virus production which followed a limited period of contact with 5-FU, was reversed when the infected cells were subsequently cultivated in 5-FU-free medium. This is in sharp contrast with the work of Kaufman and Maloney on the treatment with IUDR of herpes simplex and vaccinia viruses in tissue culture. In their experiment cultures treated for about 4 days were "cured." When given growth medium free of IUDR, no relapse was observed in the previously diseased cultures and the cells appeared healthy (Kaufman and Maloney, 1963). RSV contains RNA, while vaccinia and herpes simplex are DNA-containing viruses.

It was suggested that infection of cells with RSV, but not with other RNA viruses, might involve interaction of the virus with the host genome since RSV is a moderate virus, i.e. infection and virus production do not cause cell death (Temin and Rubin 1959). RSV can exist in cells in a provirus state (Temin 1963). Results of experiments by Temin (1963) suggest a direct interaction of RSV with host DNA. Actinomycin D exerts an inhibitory effect by complexing with DNA. Low concentrations of actinomycin D (0.1 µg/ml) reversibly inhibit production of Rous Sarcoma Virus; higher concentrations inhibit irreversibly. Concentrations of actinomycin inhibitory to RSV production do not destroy cells so that they cannot pro-

duce virus. A concentration of actinomycin 100 times greater than that required to give 95 percent inhibition of RSV production does not prevent synthesis of NDV-an RNA virus, so that the effect of actinomycin on RSV production is probably mediated through DNA.

Another group of workers (Barry et al., 1962) investigated the effects of actinomycin D on the replication of two myxoviruses; influenza virus, and Newcastle disease virus. Although both viruses contain RNA, influenza virus replication is inhibited by the antimetabolite while that of NDV is not affected. Since actinomycin D blocks the function of cellular DNA it appears that influenza virus will replicate only in cells whose DNA function is unimpaired.

Using 5-FU on both DNA and RNA containing viruses in tissue culture systems, a group of Japanese workers showed that this antimetabolite had no effect on the replication of RNA-containing viruses such as polio virus types 1, 2, and 3. It suppressed the growth of vaccinia virus, while adenovirus, which also contains DNA, showed no sensitivity to this compound. (Toyoshima et al., 1962). According to Munyon and Salzman (1962), high concentrations (10^{-3}M) of 5-FU substituted virus had the same specific infectivity as the unsubstituted virus.

Much less information has been made available on the

effects of purine antimetabolites on virus replication, since only a few purine analogues have been found effective against animal viruses. Thioguanine (2-amino-6-mercaptopurine or 6-TG) has been found to be capable of producing 50 percent or more tumour growth inhibition of the RC carcinoma (Tarnawoski and Chester, 1958). Other carcinomas are less sensitive than the RC type to 6-mercaptopurine and thioguanine.

Sartorelli and LePage (1958) observed that thioguanine inhibited the azazerine-induced accumulation of formyl glycinamide ribonucleotide (FGAR) (See Appendix). Results of their investigations on 6-TG support the concept that the incorporation of 6-TG into DNA is closely linked to the toxicity produced in normal or neoplastic cells. The analogue may also be incorporated into RNA. In a series of normal and neoplastic tissues of the rodent, 6-TG was found in the nucleosides of both DNA and RNA of susceptible tissues. It appears earliest in the nuclear RNA and, in general, is incorporated most rapidly where the turnover of RNA is fastest (LePage and Jones, 1961).

Working with Ascites cell mouse tumours, the latter group of investigators (LePage and Jones, 1961) established that susceptible tumours showed maximum response to treatment initiated at any time during the rapid-growth phase; that in susceptible tumours, a large part of the incorporation was in

DNA, and that three treatments with 6-TG were sufficient to produce an essentially maximum tumour inhibition. The tumour-inhibitory properties of 6-TG resulted from its incorporation into nucleic acids, probably specifically the DNA material. It appears that cells which make DNA-containing thioguanine remain viable for a considerable time but are unable to replicate their DNA. This is suggested as the mechanism by which thioguanine inhibits susceptible tumours. It is likely that the analogue exerts a similar effect on virus-infected cells.

In studies on purine antimetabolites and replication, Thompson et al. (1949, 1950) report the finding that 2,6-diaminopurine (DAP) decreased growth of vaccinia virus, in tissue culture, in a concentration of $5X10^{-6}M$. From another laboratory (Gifford et al., 1954), there are reports that 2-aminopurine (2-AP) was inactive as an inhibitory agent, and that 2, 6-DAP had no effect on polio virus infection of HeLa cells except at concentrations that were inhibitory to cell respiration. Brown (1952) found that 2, 6-DAP inhibited multiplication of poliomyelitis virus type 2 (MEF_1) in monkey testicular tissue culture with partial reversal of inhibition by adenine or guanine.

The observed effects of 6-mercaptopurine (6-MP) or purine synthesis <u>de novo</u> can be attributed to inhibition by 6-MP ribonucleotide of phosphoribosylamine synthesis. 6-MP is

known to be metabolized by pathways of hypoxanthine metabolism, and to interfere with the synthesis and interconversion of purine nucleotides (Brockman, 1963). The observations that natural purines affected an early step in purine synthesis de novo was extended to purine analogues by Gots and Gollub (1959) who observed that mercaptopurine and 6-thioguanine were particularly effective as inhibitors of the accumulation of 5-amino-4-imidazole carboxamide ribonucleoside by Escherichia coli B-96.

Some interesting results were obtained by Cogniaux-Le Clerc (1962) who studied the effect of 8-azaguanine on the synthesis of vaccinia virus. The antimetabolite inhibited the multiplication of vaccinia, a DNA-containing virus, although the presence of the analogue could be demonstrated only in the RNA of the infected cell. The action of 8-azaguanine on vaccinia virus synthesis was studied on chick embryo monolayers, under agar overlay or in liquid medium. The results of this study support the view that RNA must play an important part in the development of viruses whose genetic material is constituted by DNA. (Tamm, 1960; Tamm et al., 1963) Cogniaux-Le Clerc offers 3 hypotheses on the mechanism of inhibition of vaccinia virus synthesis by the analogue. First, that a very low amount of 8-azaguanine incorporates into the DNA-genetic material of the virus, giving rise to non-infectious units. Although it is doubtful that the inhibition of

vaccinia virus synthesis is relevant to this mechanism, it cannot entirely be excluded. Another possibility is that the antimetabolite might have some indirect effect on synthesis of the virus DNA. Interference with normal RNA synthesis during the lag phase prevents the onset of DNA synthesis in most cells (Harris, 1959). The third hypothesis is that the analogue inhibits primarily the synthesis of the virus proteins.

Methods and Materials

Antimetabolites

5-Fluorouracil (NSC 19893) and thioguanine (NSC 752) were obtained through Dr. J.E. Bismanis as a gift from the Cancer Chemotherapy National Service Center, National Cancer Institute, Bethesda 14, Maryland.

The required amount of the antimetabolites were weighed out exactly on a Christian Becker balance and made up to volume in a volumetric flask. The cellular antimetabolites were made up at a concentration of 10,000 micrograms per milliliter (ug./ml.) in physiological saline buffered at pH 7.2. A few drops of 5N KOH were needed to effect solution of the thioguanine. Heating in a water bath at 40°C was necessary for the complete solution of the compounds.

The solutions were then filtered through a Millipore filter, of 0.45 micron (u) pore size, fitted to a Swinny adapter and a 30 cc. syringe. A drop of the filtered solution was tested for sterility on a blood agar plate which was incubated at 37°C for 48 hours. The stock solutions were kept frozen at -18°C until required for use. From these, appropriate dilutions were made in sterile saline to obtain the desired concentrations.

Each antimetabolite was injected by deep penetration into the yolk sac by means of a syringe fitted with a 26 gauge

1½ inch needle. The entire needle was inserted into the egg through a hole drilled in the shell over the air sac region, and the inoculum was then expelled (Waddington et al., 1958). Solutions of antimetabolites were injected either immediately after introduction of the virus or 1½ hours later, as indicated.

Eggs

Source, Incubation, Preparation

Eggs were obtained at the poultry farm at the University of British Columbia, Vancouver, Canada. They were incubated in an egg incubator at 37°C and turned daily. On the tenth or eleventh day of incubation the eggs were candled in a dark room, and infertile eggs and dead embryos were discarded. The top of the embryo free of blood vessels and the area of the air sac were marked. The working area of each egg was swabbed with tincture of iodine followed by 70% alcohol before drilling, and also prior to and after injection of the virus or antimetabolite.

Allantoic Cavity Inoculation

With the use of an electric rotor drill a small hole was drilled in the shell above the embryo, care being taken not to pierce the shell membrane. A similar opening was made in the middle of the air sac region in order to prevent back

flow of the inoculum which was contained in a tuberculin syringe fitted with a 26 gauge, 3/4 inch needle. The needle was introduced at an oblique angle into the opening leading to the allantoic cavity, and the virus inoculum was injected there. After inoculation both openings were sealed with melted paraffin, and the eggs reincubated at 37°C for 33 - 36 hours.

Chorio-allantoic Membrane Inoculation (CAM-inoculation)

A small hole was drilled in the center of the air sac and a triangular area marked off on the top of the embryo. After drilling, the triangular piece of shell was lifted off with a pair of forceps, and with a sterile needle a small slit was made in the shell membrane without piercing the chorio-allantoic membrane (CAM) lying directly beneath it. By means of a large pipette bulb, gentle suction was applied at the hole in the air sac until the CAM fell away from the shell membrane. This could be detected upon candling the egg. The slit in the shell membrane was lengthened slightly, and through it the virus inoculum was deposited onto the CAM. The window was subsequently covered over with transparent tape, and the eggs were then tilted slightly to distribute the inoculum over the surface of the CAM. After incubation at 37° C for 48 hours, the membranes were collected and evaluated for virus concentration.

Red Blood Cells

Guinea pig erythrocytes were first employed in hemagglutination titrations of Newcastle Disease Virus (NDV). Blood was obtained by cardiac puncture from guinea pigs reared for the Department of Bacteriology and Immunology at the University of British Columbia. A sterile 20 ml. syringe fitted with a 20 gauge needle was used for withdrawal of blood from the animal. Approximately 10 ml. of blood could be obtained from a successful heart bleed on one guinea pig.

The blood was collected into an Erlenmeyer flask containing as anticoagulant a solution of 3.8% sodium citrate. After centrifugation at 2000 r.p.m. at 4°C for 15-20 minutes, the plasma was removed and the erythrocytes washed three times with sterile saline. The packed cells were made up to a 10% suspension in saline. For hemagglutination (HA) tests the cells were used as a 0.5% suspension in saline.

Eventually, chicken erythrocytes were used in all the HA tests. Blood was collected at the poultry farm at the University of British Columbia, and washed in the same manner as for guinea pig erythrocytes. With NDV, hemagglutination was more pronounced when these erythrocytes were used than when guinea pig red cells were employed in HA tests. Also, the hemagglutinin of vaccinia virus often agglutinates the erythrocytes of only certain species of fowls. This appears

to be a genetic characteristic, unrelated to age, sex or breed (Clark and Nagler, 1943). The hemagglutinin is not an integral part of the virus and may be separated from the virus particle.

Preparation of Virus Suspensions

Newcastle Disease Virus (NDV)

A suspension of NDV, grown on HeLa cells (9 July, 1962), and with a titer of 160 hemagglutinating units (HA units), was obtained from Dr. J.E. Bismanis approximately two months after storage at -18°C. The virus suspension was thawed just before use and kept on ice. Virus dilutions were made in sterile saline buffered at pH 7.2.

0.1 ml. of virus suspension was inoculated into the allantoic cavity of 10-11 day old chick embryos. The openings were sealed and incubation carried out at 37°C for 33-36 hours.

Harvesting of Allantoic Fluid

At the end of the incubation period the embryos were candled and dead embryos were discarded. Surviving embryos were chilled at 4°C for about four hours before harvesting the allantoic fluid. The eggs were placed on end, the shell over the air sac was removed with forceps, and the shell membrane beneath torn away with sterile instruments. Allantoic fluid was then withdrawn by means of a sterile Pasteur pipette

and collected into sterile test tubes. Fluid showing any sign of turbidity was discarded. A drop of the pooled fluid containing the virus was placed on a blood agar plate which was incubated at 37°C for 48 hours to test the sterility of the fluid. The pooled fluid was centrifuged at 2000 r.p.m. at 4°C for 15 minutes for clarification. An hemagglutination test was carried out on the suspension which was subsequently dispensed into the screw-capped tubes in 1 ml. quantities for storage at -18°C.

Vaccinia Virus

Virus was obtained from glass capillary tubes containing smallpox vaccine as manufactured by Connaught Laboratories at Toronto, Canada. Each capillary contained approximately 0.05 ml. of the vaccine. The contents of capillaries were diluted 1:10 in sterile buffered saline, and 0.1 ml. was inoculated onto the chorio-allantoic membrane of 10-11 day old chick embryos. After three passages on the CAM, the titer of the virus used in the tests was 512 HA units per ml.

Harvesting of Chorio-allantoic Membranes (CAM)

After incubation for 48 hours at 37°C, the eggs were refrigerated for approximately 4 hours before harvesting. The shell was removed to the level of the fallen CAM, and the membranes removed aseptically with forceps and scissors into sterile petri dishes containing saline. The membranes were

either homogenized immediately or stored overnight at -18°C to facilitate the grinding. Membranes were suspended in sterile buffered saline (0.5 ml. per membrane) and ground up in a Potter homogenizer. The homogenate was then centrifuged at 2000 r.p.m. for 15-20 minutes at 4°C, and the supernatant fluid containing the virus was dispensed in 1.0 ml. quantities into screw-capped tubes which were stored at -18°C. This was subsequently used as vaccinia virus suspension.

Tests with Antimetabolites and Virus

In each experiment, six embryos were used for the test and for appropriate controls as follows:

Test

0.1 ml. of virus dilution was inoculated via the allantoic cavity or the CAM of six 10-12 day embryos, followed by the antimetabolite via the yolk sac. The dose of 5-fluorouracil employed was 0.5 ml., while that of thioguanine was 0.25 ml.

Controls

0.1 ml. of virus dilution was inoculated via the allantoic cavity or the CAM of six 10-12 day embryos.

0.1 ml. of normal allantoic fluid or ground up CAM suspension was inoculated into embryos, the former via the allantoic cavity, and the latter via the CAM.

0.5 ml. of 5-FU was injected into the yolk sac of each embryo or 0.25 ml. of thioguanine by the same route.

0.1 ml. of sterile buffered saline was inoculated via the allantoic cavity or the CAM.

Untreated eggs incubated for the same period of time as those under test.

Methods of Assaying the Production of Virus-specific Material

Determination of the Hemagglutinating Titer of the Virus-containing Fluid

Hemagglutination tests were carried out in duplicate and set up in agglutination tubes with round bottoms.

Newcastle Disease Virus

Hemagglutinating activity of NDV was assayed by the pattern test described by Salk (1944), using equal volumes of serial two-fold dilutions of virus and 0.5 percent red blood cell suspension. A control tube was included containing 0.5 ml. saline and 0.5 ml. red blood cells. The racks of tubes were shaken and allowed to stand at 4°C for 45-60 minutes.

Vaccinia Virus

Smaller quantities of vaccinia virus were used, 0.25 ml. of virus suspension and an equal volume of chicken erythrocytes (Clark and Nagler, 1943). The optimum temperature for vaccinia virus hemagglutination is 37°C. (Rhodes and VanRooyen)

The racks of tubes were shaken vigoursly to obtain

even dispersal of the reagents and then allowed to stand at 37°C until the cells in the control had settled (approximately 45-60 minutes).

The endpoint of the titration was read by determining the highest dilution of virus to give agglutination of red cells as indicated by the presence of a film of red cells on the bottom of the test tube. In the absence of agglutination, the erythrocytes form a solid ring at the bottom of the test tube. The reciprocal of the virus dilution in the last tube to show complete hemagglutination was taken as the hemagglutinating titer of the virus suspension.

Determination of the Infectivity Titer of the Virus-Containing Fluid

This method was employed in determining whether vaccinia virus, harvested from embryos treated with antimetabolites retained any of its infectivity for the chick embryo.

Estimation of virus activity was carried out on the CAM of the chick embryo by Burnet's method (Burnet, 1942). The virus suspension was prepared in serial ten-fold dilutions in sterile buffered saline, and 0.1 ml. of each dilution was deposited onto the CAM of hens' eggs which had been incubated for 10-11 days at 37°C. After a further 48-72 hours incubation the membranes were excised and examined for the presence of specific foci or for thickening of the membrane

indicative of infection. Since discrete foci were not always obtained, no attempt could be made to calculate the fifty per cent infectious dose (ID_{50}) of the virus suspension.

The infectivity titer of the vaccinia virus for the chorio-allantoic membrane of the chick embryo was taken as the highest dilution of virus suspension to produce specific foci or thickening of the membrane indicative of infection. Controls were set up to account for any non-specific reaction.

Results

Tolerance of the Chick Embryo for Antimetabolites

5-Fluorouracil

The chick embryo of 10-11 days could survive a dose of 5,000 ug. of 5-FU for 48 hours at 37°C. The analogue was injected into the yolk sac of the egg, four embryos being used for each dilution of antimetabolite in determining the tolerance dose. Uninoculated controls and controls receiving only saline were also set up. The dose of antimetabolite in the tests was the maximum concentration tolerated by at least 3 out of 4 embryos for 48 hours at 37°C. This gives the maximal effective concentration of the antimetabolite.

Thioguanine

Four embryos were used for each dilution of antimetabolite in determining the tolerance dose. The chick embryo could survive 2,000 µg of thioguanine for 48 hours at 37°C. Similar controls were set up as for 5-FU.

Tests on NDV with 5-Fluorouracil

Analogue added immediately after infection of the chick embryo with virus.

Analogue added 1½ hours after infection of the chick embryo with the virus. (Shatkin, 1963)

The titer of NDV used was 640 HA units per ml. Six

embryos were employed in each test and each control group. A 10^{-2} dilution of the stock virus was used in the tests.

Results of Hemagglutination Tests when 5-FU was added at Zero Time

Chicken RBC

DILUTION OF ALLANTOIC FLUID	5-FU vs. NDV		NDV CONTROL		5-FU CONTROL	NORMAL ALLANTOIC FLUID CONTROL
1;10	+	+	+	+	-	-
1:20	2+	2+	+	+	-	-
1:40	2+	2+	2+	3+	-	-
1:80	3+	3+	3+	3+	-	-
1:160	3+	3+	3+	3+	-	-
1:320	2+	2+	2+	2+	-	-
1:640	-	-	±	±	-	-
1:1280	-	-	-	-	-	-
1:2560	-	-	-	-	-	-
Control	-	-	-	-	-	-

HA Titers: Control NDV=640 HA units/ml. of original virus suspension
Test NDV=640 HA units/ml.

There is no significant reduction in the HA titer between the test and the control

Key to Symbols +, 2+, 3+ = degree of hemagglutinating activity.
± = partial hemagglutination
- = no hemagglutination

The following results were obtained when guinea pig red blood cells were employed in the hemagglutination tests. The hemagglutination was less pronounced than with the chicken erythrocytes which were employed in subsequent HA tests.

Guinea Pig RBC

DILUTION OF ALLANTOIC FLUID	5-FU vs. NDV		NDV CONTROL		5-FU CONTROL
1:10	+	+	+	+	-
1:20	+	+	+	+	-
1:40	2+	2+	2+	2+	-
1:80	2+	2+	3+	2+	-
1:160	3+	2+	2+	3+	-
1:320	+	+	+	+	-
1:640	-	-	-	-	-
1:1280	-	-	-	-	-
1:2560	-	-	-	-	-
Control	-	-	-	-	-

HA titers: Control NDV = 640 HA units/ml.
Test NDV = 640 HA units/ml.

There is no significant reduction in the HA titer between the test and the control.

Results of Tests when 5-FU was added 1½ Hours after the Virus

DILUTION OF ALLANTOIC FLUID	5-FU vs. NDV		NDV CONTROL		5-FU CONTROL
1:10	±	+	+	+	-
1:20	2+	2+	+	+	-
1:40	2+	2+	2+	3+	-
1:80	3+	3+	3+	3+	-
1:160	3+	3+	3+	3+	-
1:640	-	-	±	±	-
1:1280	-	-	-	-	-
1:2560	-	-	-	-	-
Control	-	-	-	-	-

HA Titers: Control NDV = 640 HA units/ml.
Test NDV = 640 HA units/ml.

Chicken erythrocytes were used in the estimation of the hemagglutinating activity of the virus since they were found to give more pronounced hemagglutination.

There is no significant change in the hemagglutinating titer of the virus when the introduction of virus precedes that of the analogue by 1½ hours.

The NDV control virus gave partial hemagglutination at a titer of 1280 HA units per ml. However, this was not considered significant since the hemagglutinating titer of the virus suspension was determined on the basis of the last tube to show complete hemagglutination.

Tests on Vaccinia Virus with 5-Fluorouracil

Antimetabolite was added 1½ hours after infection with vaccinia virus. The titer of vaccinia virus used in the tests was 512 HA units per ml. Six embryos were employed in each test and control group. A 10^{-1} dilution of the stock virus was used in the tests.

Hemagglutination with Chicken Erythrocytes

DILUTION OF CAM VIRUS	5-FU vs. VACCINIA		VACCINIA CONTROL	
1:2	+	+	+	+
1:4	+	+	3+	+
1:8	2+	2+	3+	3+
1:16	2+	2+	3+	3+
1:32	+	+	3+	3+
1:64	-	-	2+	2+
1:128	-	-	+	+
1:256	-	-	-	-
1:512	-	-	-	-
Control				

HA Titers: Vaccinia Control = 512 HA units/ml.
Vaccinia Test = 128 HA units/ml.

5-FU CONTROL	UNINFECTED CONTROL
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-

There is a four-fold reduction in the hemagglutinating activity of Vaccinia virus harvested from embryos treated with 5-FU.

Tests on Newcastle Disease Virus with Thioguanine

The titer of NDV employed was 640 HA units per ml. Six embryos were used in each test and control group as before. A 10^{-2} dilution of virus suspension was used in the tests.

Results of Hemagglutination Tests when Thioguanine was added at Zero Time.

DILUTION OF ALLANTOIC FLUID	THIOGUANINE vs. NDV		NDV CONTROL	
1:10	-	-	-	-
1:20	+	+	+	-
1:40	3+	2+	2+	+
1:80	3+	3+	3+	3+
1:160	3+	3+	3+	3+
1:320	3+	3+	3+	3+
1:640	2+	2+	2+	2+
1:1280	+	+	+	+
1:2560	-	-	-	-
Control	-	-	-	-

THIOGUANINE CONTROL	NORMAL ALLANTOIC CONTROL
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-

HA Titers: Control NDV = 2560 HA units/ml.
Test NDV = 2560 HA units/ml.

There is no reduction in the hemagglutinating activity between virus obtained from treated and from untreated embryos.

Results of Hemagglutination Tests when 6-TG was Administered 1½ Hours after the Virus

DILUTION of ALLANTOIC FLUID	6-TG vs. NDV		NDV CONTROL	
1:10	-	-	-	-
1:20	+	-	-	+
1:40	3+	2+	+	2+
1:80	3+	3+	3+	3+
1:160	3+	3+	3+	3+
1:320	3+	3+	3+	3+
1:640	2+	2+	3+	2+
1:1280	+	+	+	-
1:2560	-	-	-	-
Control	-	-	-	-

6-TG CONTROL	NORMAL ALLANTOIC FLUID CONTROL
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-
-	-

HA Titers: Control NDV = 2560 HA units/ml.
Test NDV = 2560 HA units/ml.

Virus-containing allantoic fluid from 6-TG treated embryos showed similar hemagglutinating activity with virus from untreated embryos.

Test on Vaccinia Virus with Thioguanine

Untreated embryos were infected with varying dilutions of the virus suspension harvested from chorio-allantoic membranes of 6-TG treated embryos. 0.1 ml. of the suspension was inoculated onto each CAM, using 4 eggs per dilution (10^{-1} - 10^{-12}). Infectivity was determined by the number of pocks on the membrane, or by generalized thickening of the CAM.

Untreated Embryos

VIRUS DILUTION	AVERAGE NO. of POCKS	Pfu/ml.
10^{-1}	Much thickening of CAM	—
10^{-2}	68	6.8×10^4
10^{-3}	3.0	3.0×10^4
10^{-4}	0.5	5.0×10^4
10^{-5}	—	

Average Number of Pfu = 4.9×10^4 Pfu/ml.

TG-Treated Embryos

VIRUS DILUTION	AVERAGE NO. of POCKS	Pfu/ml.
10^{-1}	Thickening of CAM	—
10-2	64.0	6.4×10^{-4}
10^{-3}	4.0	4.0×10^{-4}
10^{-4}	Generalized thickening	—
10^{-5}	Generalized thickening	—

Average Number of Pfu = 5.2×10^{-4} Pfu/ml.

There was no recognizable reduction in the infection of membranes from both groups. At higher dilutions generalized thickening of the CAMs were evident.

Discussion

The antimetabolite 5-fluorouracil, (5-FU), is a structural analogue of thymine which is an essential component of DNA. Since the carbon-fluoride bond is strong, the substitution of a fluorine atom for the hydrogen in the 5 position of uracil makes 5-FU an interesting antimetabolite. A theoretical reason advanced for the synthesis of 5-FU was that the van der Waal radius, (1.35Å), of the fluorine atom is very close to that of hydrogen, (1.2Å), and might cause the compound to block the enzymatic reaction to DNA synthesis with greater efficiency than other substituents on the 5- position. (Welch, 1961) However, it was realized more recently (Sadler, 1963), that there is a gradation of antiviral activity with increasing van der Waal's radii of the substituents and that the fluorine atom, which has the smallest radius apart from hydrogen, decreases anti-viral activity the least. Iodouracil and bromouracil undergo dehydrogenation although 5-FU does not.

5-FU is metabolized to 5-fluorouridine (FUR) and then to fluorouridine monophosphate (FUMP), or fluorouridylic acid (See Appendix, Fig. 2.). FUMP may block the incorporation of the normal metabolite UMP into RNA, or it may be incorporated into RNA to form a fraudulent fluorine-containing RNA. (Burchenal and Oettgen, 1960; Salzman et al., 1962). The analogue

5-FU may also replace thymine in DNA. If incorporated, a structural analogue of a metabolite might cause lethal synthesis, i.e. the production of inactive viral progeny. (Tamm and Eggers, 1963). Sadler (1963) also shares the opinion that the incorporation of unnatural bases or nucleotides into virus nucleic acid should necessarily impair replication. For the RNA-containing viruses, it would seem important to inhibit specifically the synthesis of RNA without affecting the metabolism of DNA.

On the contrary, other workers report that 5-FU has no inhibitory effect on RNA viruses. Munyon and Salzman (1962) report that 5-FU is effectively incorporated into poliovirus RNA. The 5-FU substituted virus had the same specific infectivity as the unsubstituted virus. The analogue was found to have no inhibitory effect on NDV grown in HeLa cells (Simon, 1961), and according to Kaufman, (1963) 5-FU is incorporated into nucleic acids and has no therapeutic anti-viral activity. The conversion product of 5-FU, FUDR is said to have no inhibitory effect on RSV (Rich et al., 1962).

The finding in my experiments that no reduction in hemagglutinating activity was obtained when NDV-infected embryos were treated with 5-FU, may probably be explained on the basis that, if the analogue was incorporated into RNA, the resulting virus was not altered with respect to hemagglutinating activity. The virus of NDV agglutinates

erythrocytes of guinea pigs and fowls. Hemagglutination is indicative of the presence of virus particles. It is probable that if a fraudulent RNA is formed, it is nevertheless replicated and produces infectious virus capable of hemagglutinating these erythrocytes. The antimetabolite was originally administered to the embryos immediately after infection with the virus via the allantoic cavity. In studies on the replication of the DNA virus, vaccinia, Shatkin (1963), and (Shatkin et al., 1963), made the observation that viral DNA formation commences within $1\frac{1}{2}$ hours after infection. Although this expressed the situation in a DNA virus, which, like NDV, replicates in the cytoplasm of the cell, it was felt that it would be interesting to observe whether 5-FU, added $1\frac{1}{2}$ hours after infection, would have any effect on an RNA virus. However, there was no difference in the hemagglutinating activity of NDV under these conditions.

In DNA synthesis, (Appendix, Fig. 2.) the normal sequence of events from uracil is via deoxyuridine (UDR), deoxyuridine monophosphate (DUMP) and thymidylic acid (TMP). When 5-FU is used it is converted to FUDR and eventually to fluorodeoxyuridine monophosphate (FDUMP) which blocks the methylation of DUMP to form TMP. (Cohen et al., 1958; Salzman et al., 1963). The FDUMP is apparently not incorporated into DNA.

Some inhibition was obtained with 5-FU and vaccinia virus as evidenced by the results of the hemagglutination tests;

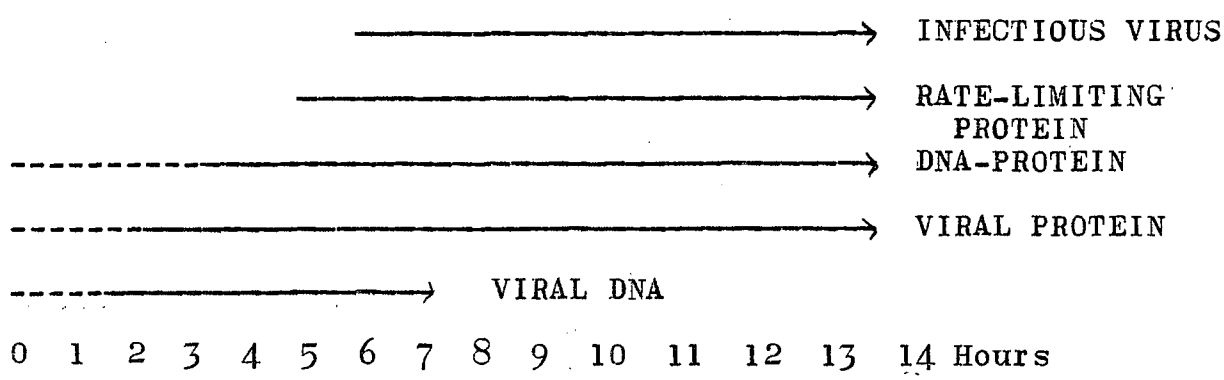
total or complete inhibition was not obtained. There is a four-fold reduction in the hemagglutinating activity of vaccinia virus when the 5-FU was administered to the chick embryo 1½ hours after the virus. It is possible that the series of events mentioned above takes place. The fact that complete inhibition was not obtained may depend on several factors, among which are: the strain of embryo treated, the stage of development of the embryo when the compound is introduced, the ability of the drug to penetrate into the embryonic cells, and the presence of protective mechanisms. Other possibilities will be discussed in a later section.

Some of the knowledge of the replication of vaccinia virus was derived from the use of the metabolic inhibitor FUDR (Salzman, 1960) which totally blocks the conversion of UDR to TMP and thus the formation of DNA. FUDR is a metabolic derivative of, and can be hydrolysed to, the free base 5-FU. Fluorouracil analogues then, may have these effects:

1) The inhibition of thymidylic acid synthesis.
2) The incorporation of FUMP into RNA to give a fraudulent RNA.
3) They may block the incorporation of UMP into RNA.

The events in the sequential synthesis of viral components in HeLa cells infected with vaccinia virus are summarized in the following chart by Shatkin (1963) and Salzman et al. (1963).

Formation of Vaccinia Virus Components (Chart and description following are from Shatkin, 1963)



Viral DNA formation commences within $1\frac{1}{2}$ hours after infection and is completed at $6\frac{1}{2}$ - 7 hours.

Viral proteins detectable by the indirect immunological method, are also formed within $1\frac{1}{2}$ - 2 hours after infection, but are synthesized throughout the infectious cycle.

"Rate-limiting protein" necessary for infectious virus formation is synthesized less than 1 hour before maturation.

The association of viral DNA and protein begins as early as 2 hours after infection.

Whereas viral DNA synthesis appears to be complete at 7 hours, the amount of DNA associated with viral protein continues to increase throughout infection.

Infectious virus is formed beginning at 5 - 6 hours and is complete at 13 - 14 hours after infection.

In studies on vaccinia virus synthesis in HeLa cells, FUDR at 10^{-6} M was added to cultures at varying times after infection and all cultures were sampled and titrated at 25 hours. Any virus formed after the addition of the inhibitor must contain DNA synthesized prior to the time the inhibitor was added, since the effect of FUDR in blocking DNA synthesis is believed to be immediate (Salzman et al., 1963).

According to Joklik (1962), when FUDR is added to infected HeLa cells at any time up to $2\frac{1}{2}$ hours after infection, no vaccinia virus is formed in these cells subsequently after 24 hours; therefore, no viral DNA was synthesized before $2\frac{1}{2}$ hours after infection, i.e. DNA synthesis was inhibited by the antimetabolite FUDR. The antimetabolite FUDR does not inhibit the incorporation of preformed thymidine derivatives into DNA, but there is so little of these compounds present in the intracellular pool of HeLa cells, that in practice, synthesis of DNA, and therefore of virus, stops immediately on the addition of FUDR to the cells. When the analogue is added to the cells 6 hours after infection with virus, the full complement of virus is produced, showing that it is in this period between $2\frac{1}{2}$ and 6 hours after infection that sufficient viral DNA is formed for incorporation into mature virus. These results generally agree with that of Salzman et al., (1963) and Shatkin (1963), except that the latter groups find that viral DNA formation commences within $1\frac{1}{2}$ hours after in-

fection.

Since FUDR and the free base 5-FU to which it can be hydrolyzed, both block thymidylic acid formation (Hartmann et al., 1961) then the fact that only partial inhibition of virus replication was achieved may mean that:

1) The effect of 5-FU was not immediate.
2) Some viral DNA is probably synthesized before 1½ hours.
3) A larger dose of antimetabolite is needed for complete inhibition.
4) 5-FU is not a very effective inhibitor since it only partially blocks DNA synthesis in cells of the CAM of the chick embryo.
5) There is a considerable amount of thymidine derivatives present in the nucleotide pool of the chick embryo chorio-allantoic cells.

The question whether viral protein formation depends on the synthesis of viral DNA was examined by Shatkin (1963). He made use of an immunological method designed to detect viral proteins in the absence of virus maturation. The synthesis of immunologically detectable viral proteins in HeLa cells infected with vaccinia virus was observed within 2 hours after infection. When DNA synthesis in infected cells was blocked by 10^{-6} M FUDR, the formation of viral proteins continued. The rate of viral protein synthesis in the presence of FUDR was maximal for the first 4 - 6 hours after infection

and continued at a diminished rate during the latter 8 - 10 hours of the infectious cycle. The formation of vaccinia proteins later in the infectious cycle as well as virus maturation are unaffected when the analogue is added after the synthesis of viral DNA. (Salzman et al., 1963)

Adenovirus, like vaccinia virus, is a DNA-containing virus. However, adenovirus DNA is believed to replicate in the nucleus of the infected cell, and the DNA of vaccinia virus in the cytoplasm. FUDR was used in the study of the role of DNA synthesis in the replication of type 2 adenovirus in suspension cultures of KB cells. When the analogue at 10^{-6} M was added to adenovirus-infected cultures at times up to 7 hours after infection, it stopped DNA synthesis in infected and uninfected cells; it permitted the synthesis of non-viral protein, RNA and acid-soluble nucleotides in these, and it prevented virus replication in infected cells. It can be inferred from this that DNA synthesis is essential for adenovirus multiplication, (Green, 1962 a) and that formation of adenovirus-specific protein is directly dependent on the synthesis of viral DNA (Flanagan and Ginsberg, 1961). When inhibitor (FUDR) was added at different times from 8 - 21 hours after infection, increasing amounts of virus were formed. It seems that adenovirus DNA is made from 7 - 8 to about 21 hours after infection. Intracellular virus is synthesized from 13 - 14 hours to about 28 hours after infection. (Green,

1962 b). The time differences in the events in the sequential synthesis of viral components are reflected in these findings. The synthesis of adenovirus components lags behind that of vaccinia virus. Such information is valuable because it gives some indication of the most appropriate time to add inhibitor, depending on the system under study.

In 1962, the antiviral agent, 5-iodo-2'-deoxyuridine (IUDR) was found to cure corneal infection, in rabbits, caused by herpes simplex viruses. (Kaufman, et al., 1962 a, b; Kaufman, 1963). This antimetabolite inhibits the phosphorylation of thymidine and the polymerization of thymidylic acid into DNA. (Delamore and Prusoff, 1962). IUDR may also be incorporated into an abnormal and presumably non-functional DNA which may be no longer infectious. The analogue FUDR acts at an earlier site and inhibits thymidylate synthetase rather then the phosphorylase. These two antimetabolites produce the same overall effect though having different sites of action.

It might be expected that purine analogue would interfere with the metabolism of nucleic acids, and thus might seem likely to have secondary consequences on protein formation. The close relationship between nucleic acid and protein biosynthesis has suggested that incorporation of analogues into polynucleotides could result in inhibition of protein synthesis or the formation of fraudulent proteins via fraud-

ulent RNA (Henderson and Mandel, 1963).

Certain purine analogues are known to mimic the natural purines in inhibition of the de novo pathway of purine synthesis in microorganisms and in mammalian cells. (Brockman, 1963) The reactions of purine bases with phosphoribosylpyrophosphate to form ribonucleotides are catalyzed by nucleotide pyrophosphorylases among which there may be species differences. After the ribonucleoside monophosphate is formed (i.e. the ribonucleotide), it may or may not be further phosphorylated to the di - and triphosphates. In experiments with 6-thioguanine (6-TG) the monophosphates predominate, but di- and triphosphates are also made. (Moore and Le Page, 1958)

Formation of deoxyribonucleotides is necessary for the action of some antimetabolites, and for those which are incorporated into DNA. This may occur by reduction of the ribonucleotide, by transdeoxyribosidation or by phosphorylation of administered deoxyribonucleosides (i.e. IUDR). The deoxyribonucleotide of TG is incorporated into DNA (Le Page and Jones, 1961); even natural purine deoxyribonucleotides are present in cells in only very small amounts compared to those of the pyrimidines.

In general, the purine and pyrimidine antimetabolites undergo the same types of catabolic reactions as do the natural compounds. Purines are oxidized at the 2, 6, and 8

positions; deamination may also occur. Incorporation of certain purine and pyrimidine analogues into nucleic acids has been clearly established, (Matthews, 1958) and appears to take place by routes characteristic of nucleic acid synthesis. Antimetabolites which are incorporated into nucleic acids of mammalian cells usually are also incorporated into microbiological systems and viruses. There has been considerable variation observed in the total amount of bases replaced by the analogues. The extent of the antimetabolite incorporated varies with the metabolic activity of the system. In viral systems where cell proliferation is rapid, the analogue may substitute completely for a normal component (Litman and Pardee, 1956). Antimetabolites incorporated into nucleic acids are not necessarily uniformly distributed as the normal components.

No purine nucleotides are known to inhibit nucleotide biosynthesis de novo. However, it is believed that they may act to inhibit nucleotide metabolism. 6-TG is believed by some investigators (Le Page and Jones, 1961) to be similar to or identical with 6-mercaptopurine, (6-MP) in its metabolic effects because of the similarity of the two structures. Although there is still little agreement as to the mechanism of its inhibitory effects, at the present time the most widely held view is that 6-MP in the form of its ribonucleotide inhibits the conversion of inosinate to either adenylosuccinate or xanthylate or both. (Appendix, Fig. 3.). Whether or not

6-TG exerts the same action is still in doubt. 6-TG is probably incorporated into DNA or RNA, a biochemical event which probably produces toxicity to the cells, or it may act by inhibiting the synthesis or the function of nucleotide coenzymes concerned in glycolysis and respiration. (Laszlo et al., 1961). Marked differences in the toxicities of these two compounds have led to the discovery of biochemical differences between them, contrary to previous belief.

In the experiment undertaken, 6-TG produced no inhibition in cells infected either with NDV or vaccinia virus. This observation, although not in accordance with other reports on the tumour-inhibitory properties of 6-TG (Sartorelli and Le Page, 1958; Le Page and Jones, 1961) and with the result anticipated in this experiment, is not surprising since it is known that alternate pathways of purine and pyrimidine metabolism exist. (Henderson and Mandel, 1963). For complete inhibition of growth therefore, all alternative pathways must be inhibited. Purine nucleotide metabolism is more complex than that of pyrimidine nucleotides, and although purine analogues have been shown to inhibit tumour growth, the situation in a tumour system is not necessarily in parallel with that of viral replication. Biological effects resulting from the incorporation of purine and pyrimidine analogues into nucleic acids have been inconsistent. Although some clear-cut relationships exist between incorporation and altered biochemical

functions, there are many examples where altered nucleic acids seem to behave like normal nucleic acids. If the substitution of the analogue takes place at a site in the nucleic acid chain which normally is a template for the active site of a particular enzyme, an important change in the structure of the site may be produced leading to impairment of the corresponding biological function. However, if substitution takes place at a different site, the function of the enzyme may be unaffected so that the antimetabolite may actually support the biochemical reaction. Therefore, while in many cases base analogue incorporation reduced infectivity, considerable infectivity still has been reported.

Part II

The Effects of a Pyrimidine Antimetabolite on Vaccinia Virus in Chick Fibroblast Tissue Culture

Part II of this thesis is the following report based on research undertaken at the University of California, at Berkeley, and which is relevant to the experimental work pursued at the University of British Columbia.

Introduction

During the course of some experimental work on the effects of a purine and a pyrimidine analogue on Newcastle Disease virus (NDV) and vaccinia virus, the author became interested in the recently published reports of that time on the therapeutic effect of 5-iodo-2'-deoxy uridine (IUDR) on herpes simplex virus infection in vivo (Kaufman and Maloney, 1962 b) and on vaccinia virus infection (Kaufman et al., 1962 a). Since it was not possible to pursue it further at that time, the author waited for the opportunity to try an experiment of this nature.

In this experiment an attempt was made to study the effect of IUDR on vaccinia virus in chick fibroblast (CF) monolayer cultures. The vaccinia virus employed in the test produces cytopathogenic changes in CF monolayers in about 48-60 hours after infection. The cytopathogenic effect (CPE) may be seen as the rounding up of cells, cell fragments, giant cells and degeneration of the cell sheet.

Materials and Methods

Chick fibroblast monolayer cultures were prepared in a nutrient medium (5% lamb serum and 95% nutrient solution composed of 0.5% lactalbumin enzymatic hydrolysate in modified Earle's balanced salt solution).

Ten-fold dilutions of stock vaccinia virus were made in modified Hanks' maintenance medium (MH). When cell monolayers were complete, 3 oz. prescription bottles were infected with 0.2 ml. of appropriate virus dilution. Virus was allowed to adsorb for 1 hour at 37°C with occasional tilting of the bottles to facilitate the spread of the virus inoculum over the cell sheet. At the end of this time the cultures were overlaid with 6 - 8 ml. MH. Tubes were infected with 0.1 ml. of MH. Uninfected controls were always included.

For plaque assay, bottles were overlaid with methylcellulose and incubated for 4 days, at 37°C. 0.1% crystal violet in 20% alcohol was used to stain the monolayers to examine for the presence of plaques.

When CPE was observed, the fluids were removed and tested for presence of virus by infecting monolayer cultures with varying dilutions of the fluid and observing these for CPE after suitable incubation.

IUDR was added to MH in concentrations of 100 mg./ml.

and 1 mg./ml. The antimetabolite was added to the medium just before use.

Cultures exhibiting CPE were treated with medium containing IUDR. The medium was changed daily because the breakdown products of IUDR, such as iodouracil, to some extent inhibit the antiviral activity of the parent compound. Control cultures were treated in an identical manner with medium free of IUDR.

After treatment with the antimetabolite for 24 hours and 48 hours, fluids were removed and tested for CPE on other CF monolayers.

Some cultures were treated with IUDR for 24 hours before infection with virus.

Results

The first set of monolayers prepared were infected after about 90 hours of incubation since there were no complete monolayers until this time. Dilutions of virus of 10^{-1} to 10^{-6} were used. After an additional 72 hours' incubation cell sheets were beginning to fall off the glass surface. In some of the control (uninfected)tubes, intact cell sheets could be seen, but in others the cells were also beginning to fall off the glass surface. The culture was 7 days old at this time.

48-hour CF monolayers were infected with vaccinia virus dilutions of 10^{-1} to 10^{-6} for observation of the CPE. Bottle cultures were infected for assay of the stock virus. The results of the plaque assay are shown in Table 1.

Monolayers infected with virus as in the above began to show CPE after 48 - 60 hours. Cultures infected with 10^{-1} and 10^{-2} virus dilutions exhibited definite and pronounced CPE, rounding up of cells, degeneration of the cell sheet at the edges, cell debris, and fragments of cells. The cell sheets were no longer continuous. At th 10^{-3} and 10^{-4} dilution CPE was just starting. No changes were evident at the higher dilution except that cell sheets were beginning to deteriorate in some of these as well as in the control tubes.

Infected cultures were treated with 100 mg./ml. of IUDR. The CPE was apparently arrested after 24 hours treatment. After 48 hours there was no progression of the cytopathic changes. In untreated controls the cell sheets were almost completely destroyed. The CPE was very extensive.

Another set of cultures was infected with varying dilutions of vaccinia stock virus. 48 - 60 hours later cytopathogenic changes were obvious in cultures. Infected cultures were treated with MH containing 1 mg./ml. IUDR. The results are shown in Table II.

Slight CPE was observed in cultures exposed to IUDR before infection with the virus.

Aliquots of fluids from infected and treated cultures were tested for CPE on other monolayers. Fluid from untreated cultures showed very clear cut and extensive CPE when tested on other monolayers. Samples of fluid from 24 hour and 48 hour treated cultures exhibited very slight CPE.

Table I

Plaque Assay of Stock Vaccinia Virus

Virus Dilution	No. of Plaques	Average	Pfu/ml.
10^{-1}	152;110	131.0	6.65×10^3
10^{-2}	35;38	36.5	18.3×10^3
10^{-3}	5;12	8.5	42.5×10^3
10^{-4}	3;0	1.5	75×10^3
10^{-5}	0;2	1.0	50×10^3
10^{-6}	0;0	0	0

Titer of Virus = 4×10^4 Pfu/ml.

Table II

Vaccinia Virus on Chick Fibroblasts

Virus Dilution	Observation Before Treatment	IUDR 1 mg./ml. 24 hrs.	IUDR 1 mg./ml. 48 hrs.
Undiluted	Extensive CPE	CPE Progressed slightly	No further CPE
10^{-1}	Pronounced CPE	Slight CPE	No CPE
10^{-2}	Some CPE evident	No CPE	No CPE
10^{-3}	Extensive CPE	Slight CPE	Slight CPE
10^{-4}	Slight CPE	No further CPE	No CPE
10^{-5}	No CPE	No CPE	No CPE
10^{-6}	No CPE	No CPE	No CPE

Discussion

With the vaccinia virus used, cytopathogenic changes were not evident until 48 - 72 hours after infection. This necessitated a waiting period longer than anticipated before treatment of virus-infected cell layers with IUDR. This created a problem in that cell sheets by this time were quite old and fragile (5 - 7 days), so that before treatment was complete, cell sheets were beginning to deteriorate and to fall off the surface of the glass.

At first, the use of a 1:200 dilution in nutrient medium of CF cells for setting up cultures did not provide complete monolayers in 48 hours. In subsequent preparations a 1:100 dilution of cells was used instead; this produced luxuriant monolayers within 48 hours.

In cell cultures infected with vaccinia virus, distinct cytopathogenic changes could be seen at 60 hours after incubation. Changes were manifested as the rounding up of cells, cell fragments, giant cell formation and loss of continuity of cell sheets. Rounded cells were abundant. In most untreated cultures cell sheets were completely destroyed 4 - 5 days after infection; treated cultures survived for about 7 days.

In the cultures treated with IUDR, the CPE did not appear to progress any further after 24 - 48 hours of treat-

ment. When fluid from control and treated cultures were tested, less CPE was caused by the latter.

The aromatic ring in IUDR is halogenated in position 5. Its structural formula is given in figure 1. This pyrimidine antimetabolite acts to inhibit the phosphorylation of thymidine and the polymerization of thymidylic acid into DNA. It may also be incorporated into an abnormal and presumably non-functional viral DNA which is rendered uninfectious. It is probable that IUDR not only prevents the infection of cells which have been previously free of virus, but perhaps is capable of stopping the synthesis of virus in cells already infected.

Summary

The pyrimidine antimetabolite, 5-fluorouracil was found to inhibit vaccinia virus replication in the chick embryo but was ineffective against Newcastle disease virus.

Thioguanine, a purine analogue, had no inhibitory effect on either virus. The assay methods employed were the hemagglutination test and the infectivity of the virus for the chick embryo.

It was possible to demonstrate to some extent that iododeoxyuridine can arrest the cytopathogenic changes in chick fibroblast cells in tissue culture, following vaccinia virus infection. This pyrimidine analogue seems capable of preventing major CPE in cells treated with IUDR before exposure to the virus. The antimetabolite was effective at concentrations of 100 mg. per ml., and 1 mg. per ml. when the drug-containing medium was changed daily.

Major differences in the metabolism of the various antimetabolites suggest differences in their mechanisms of action. Among the possible areas of inhibition are blocking of the incorporation of the normal metabolite, incorporation of the analogue into RNA or DNA to form fraudulent nucleic acids.

Also, the antimetabolite may inhibit synthesis of

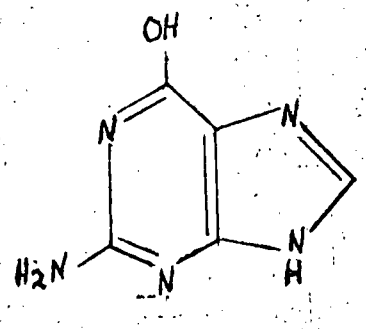
the normal metabolite or may block nucleotide metabolism.

Pyrimidine and purine analogues may inhibit enzymatic reactions, for example, the enzymatic phosphorylation of the normal bases.

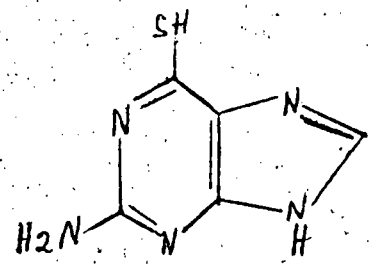
Finally, inhibition of protein synthesis may be cited as a possible mechanism of action because of the close relationship between nucleic acid and protein synthesis.

Correlations between systems must be interpreted with caution; despite many similarities in the actions and metabolism of antimetabolites in biological systems studied, differences have been uncovered.

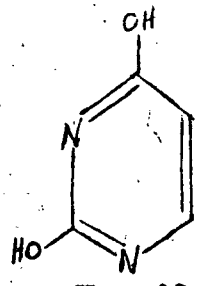
Appendix



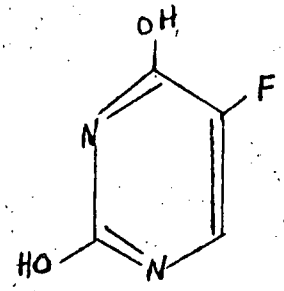
Guanine



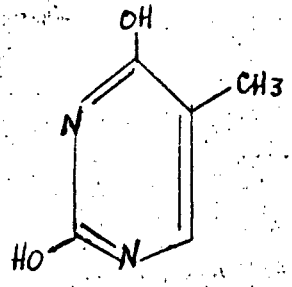
Thioguanine (6-TG)



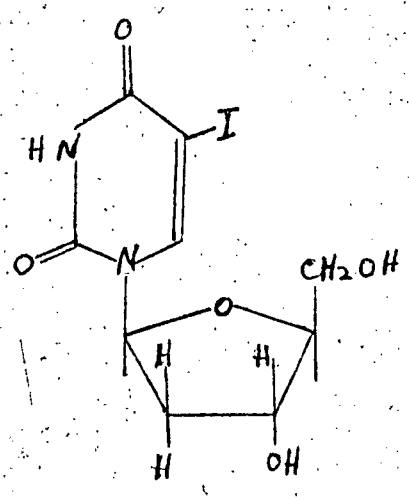
Uracil



Fluorouracil (5-FU)



Thymine



5-iodo-2-deoxyuridine (IUDR)

FIGURE I.

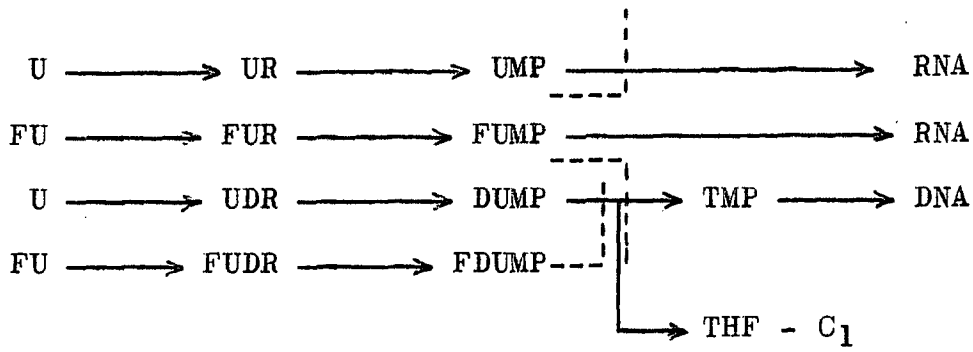


Figure 2. Formation of RNA and DNA

Key to Symbols

U - uracil

UR - uridine

UMP - uridine monophosphate

RNA - ribonucleic acid

FU - fluorouracil

FUR - fluorouridine

FUMP - fluorouridine monophosphate

UDR - deoxyuridine

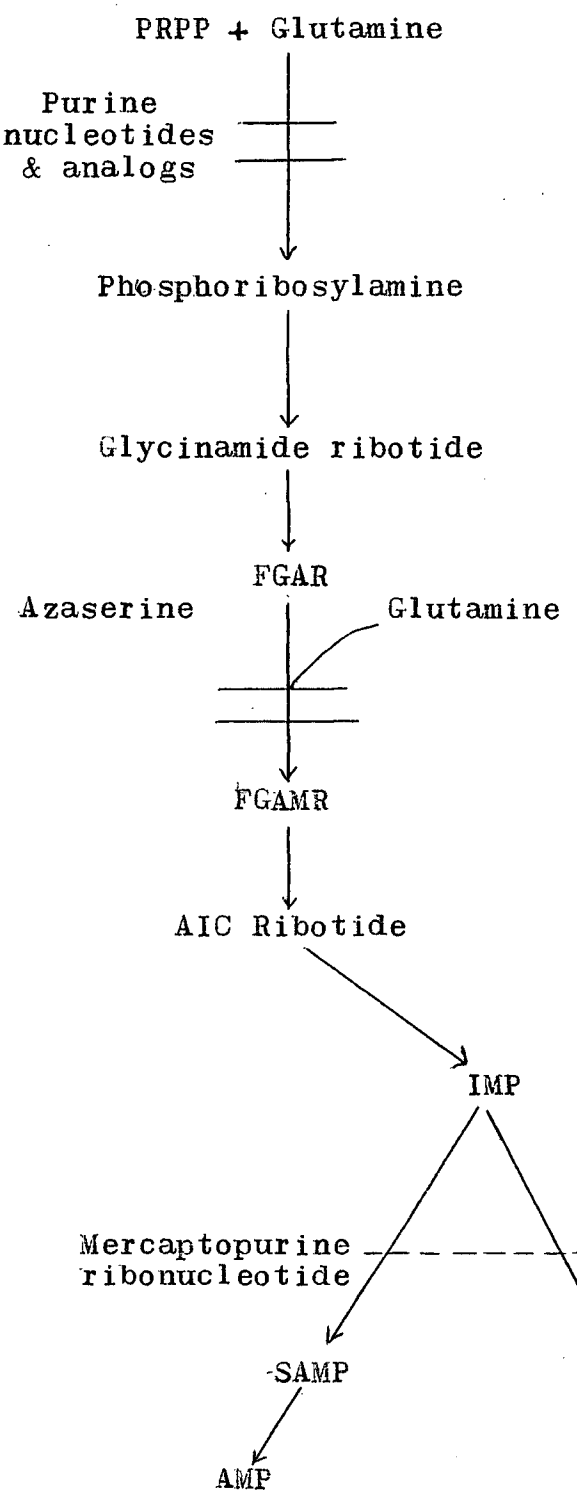
DUMP - deoxyuridine monophosphate

TMP - thymidine monophosphate

DNA - deoxyribonucleic acid

FUDR - fluorodeoxyuridine

FDUMP - fluorodeoxyuridine monophosphate



Key toSymbols

PRPP - 5-phosphoribosyl-1-pyrophosphate

FGAR - formylglycinamide ribonucleotide

FGAMR - formylglycinamidine ribonucleotide

AIC - 5-amino-4-imidazole carboxamide

SAMP - adenylosuccinic acid

XMP - xanthylic acid

IMP - inosine monophosphate

AMP - adenosine monophosphate

GMP - guanosine mono phosphate

Figure 3. Known Sites of Action of Purine Analogs. (Brockman, 1963)

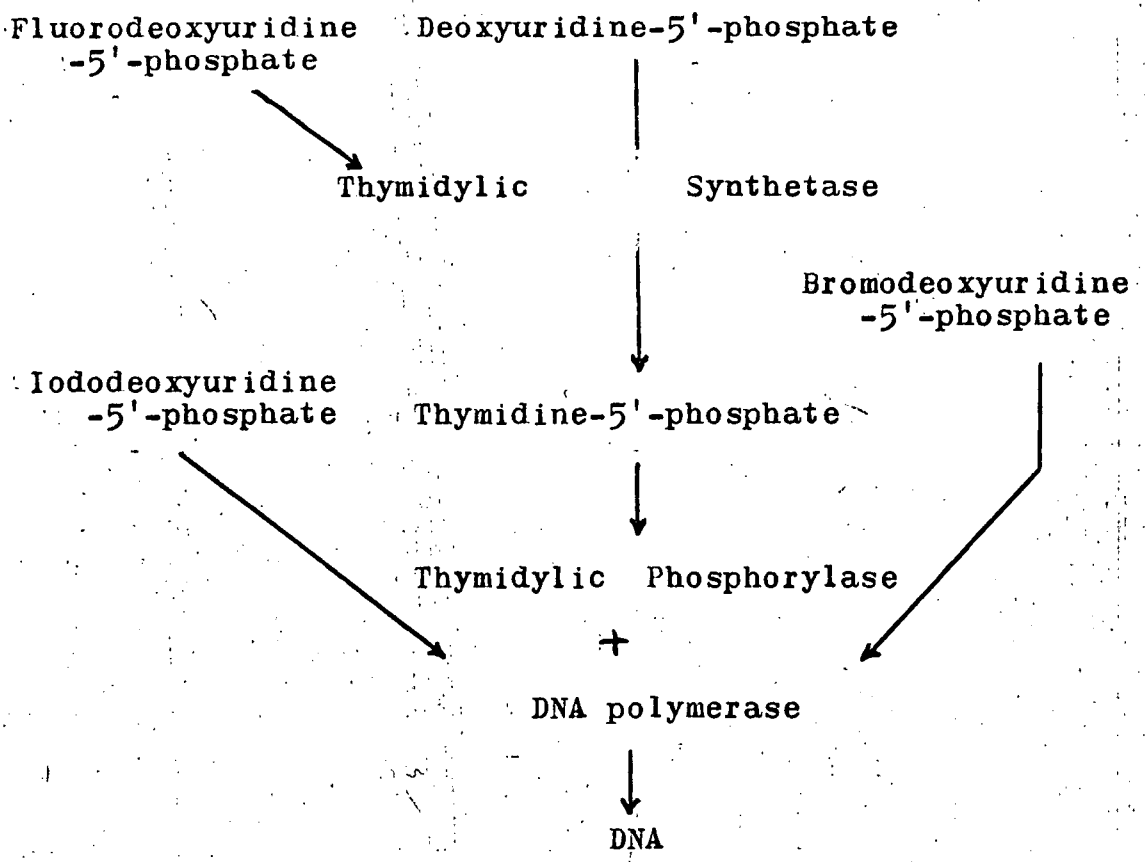


Figure 4. Site of Action of Pyrimidine Analogues

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