Plaque Inhibition Test for Detection of Specific Inhibitors of DNA Containing Viruses. (26560)

ERNEST C. HERRMANN, JR.** (Introduced by Edward J. Foley)*
Department of Chemotherapy and Infectious Diseases, Schering Corporation, Bloomfield, N. J.

Recently it has been reported that 5-bromo- and 5-fluoro-2'-deoxyuridine (BDU and FDU) not only interfered with normal deoxyribonucleic acid (DNA) biosynthesis in animal and bacterial cells but also specifically inhibited viral DNA synthesis(1-3). Using the agar-diffusion plaque-inhibition test(4), it was discovered that still another member of this series, 5-iodo-2'-deoxyuridine (IDU), was a potent inhibitor of plaque formation by vaccinia and herpes simplex viruses which are considered to contain DNA. Plaque formation of ribonucleic acid (RNA) containing West Nile (WN) and Newcastle disease (ND) viruses was not inhibited. The usefulness of the plaque inhibition test for detection of such inhibitors was examined in the present study. In addition to testing the halogen derivatives of deoxyuridine a variety of other related compounds was examined, some of which might be expected to have an influence on nucleic acid biosynthesis. Data are included to show that the agar-diffusion test also is useful for detecting thymidine reversal of IDU and BDU plaque inhibition.

Materials and methods. Preparation of agar overlay, chick embryo cell cultures in Pyrex baking dishes, infection of these cultures with vaccinia, herpes, WN and ND viruses, the strains used, subsequent staining of the cells with iodonitrotetrazolium† chloride to observe plaques and detection of zones of plaque inhibition previously have been described(4). Both growth (lactalbumin-calf serum) and agar overlay (lactalbumin-yeast extract) media were slightly modified by replacing the bicarbonate buffer with Tris-HCl buffer.‡ In addition, 90 mm plastic Petri dishes (tissue culture type, Falcon Plastics) also were used with proportional amounts of cells, media, etc. as previously described for the baking dish cultures. *Test compounds* were stored at 4°C, and at the time of the test a small amount of each was deposited directly on the surface of a freshly infected agar overlay culture. It was estimated that the quantity tested by this technic was no less than 200 μg. Generally, 200 μg can be considered a high level of material in this type of test. When plaque inhibition was observed, the compound was retested at known concentrations in a volatile solvent, usually dimethylformamide. One-quarter inch filter paper discs were impregnated with 0.02 ml of solution and then air dried. The discs then were arranged on the surface of freshly infected agar overlay cultures.

Results and discussion. The data show (Table 1) that as little as 1-2 μg of IDU or BDU inhibited plaque formation by vaccinia and herpes simplex viruses while 200 μg did not influence WN and ND viruses. Plaque inhibition was not observed with any of the other compounds including 3 samples of aminopterin. This compound has been reported, however, as an inhibitor of normal DNA biosynthesis and a specific inhibitor of DNA containing polyoma virus in mouse embryo cell cultures(1). There are numerous possible reasons for this apparent discrepancy including differences in viruses, host cells, media, and technic. BDU, however, was active in both systems. FDU, on the other hand, has been reported as a specific

* With technical assistance of Carol Leick, Judith Flavell and Ann Halpin.

** Present address: Mayo Clinic, Rochester, Minn.

† In the original reference(4) this compound was spelled incorrectly as indonitrotetrazolium chloride. The correct name of the compound is 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride or INT.

‡ To a 100 ml 2M solution of tris-hydroxymethylaminomethane was added 2M HCl to bring the pH to 7.6, and then the volume was brought to 200 ml. This stock solution was added at the rate of 1.8 ml to each 100 ml of media. A small amount of bicarbonate also was present (1 ml per 100 ml of media of a 7.5% $NaHCO_3$ solution).

TABLE I. Effect of Various Compounds on Virus Plaque Development.

Compound and source	Concentration	Plaque-free zone diameter (mm) Vaccinia	Herpes simplex	West Nile	Newcastle
5-Iodo-2'-deoxyuridine (NBC)	200 μg/disc	65	60	0, 0	0, 0
Idem	20 "	55, 53, 47	50		
"	10 "	28	30		
"	5 "	26			
"	2 "	22			
"	1.25 "	18	20		
5-Bromo-2'-deoxyuridine (NSC 38297)	200 "	60–70	45, 50	0	0, 0
Idem	20 "	58	35, 35	0	0, 0
"	10 "		30		
"	5 "		25		
"	2.5 "		trace		
5-Fluoro-2'-deoxyuridine* (NSC 27640)	>200 μg	0, 0, 0, 0	0, 0, 0	0	0
Aminopterin (NSC 739)	>200 "	0, 0, 0	0, 0, 0	0	0
" (Lederle)	>200 "	0	0	0	0
" (CCBR)	>200 "	0	0	0	0
Deoxyuridine (CCBR)	>200 "	0, 0, 0	0, 0, 0	0	0
5-Bromouridine (NBC)	>200 "	0, 0	0, 0	0, 0	0
Uridine (NBC)	>200 "	0	0, 0	0	0, 0
5-Hydroxyuridine (NBC)	>200 "	0	0	0	0
6-Azauridine (NBC)	>200 "	0	0	0	0
Uridine-5-phosphate, Na salt (NBC)	>200 "	0	0	0	0, 0
Iso-propylidene uridine (NBC)	>200 "	0	0	0	0
Thymidine (CCBR)	>200 "	0, 0, 0	0, 0, 0	0	0
5-Iodouracil (CCBR)	>200 "	0	0, 0	0	0, 0
5-Bromouracil (CCBR)	>200 "	0, 0	0, 0	0	0
6-Azauracil (NSC 3425)	>200 "	0	0	0	0
5-Fluorouracil (NSC 19893)	20 μg/disc	0, 0	0, 0	0	0
5-Bromo-1-methyl-hydrouracil (NSC 42058)	20 "	0	0	0	0
6-Propylthiouracil (CCBR)	>200 μg	0	0	0	0
Thymine (CCBR)	>200 "	0, 0	0, 0	0, 0, 0	0
Deoxyadenosine (CCBR)	>200 "	0	0, 0	0	0
Adenosine (CCBR)	>200 "	0	0	0	0
Adenine (CCBR)	>200 "	0	0	0	0
Orotic acid (CCBR)	>200 "	0, 0	0	0	0, 0

NBC = Nutritional Biochemicals Corp. NSC = Cancer Chemotherapy Nat. Service Center. CCBR = California Corp. for Biochemical Research.

* A new sample of this compound (Batch No. 30—Hoffmann-LaRoche) was recently received and tested at 200, 20 and 2 μg per test disc against vaccinia and herpes simplex viruses. Plaque suppression was again not observed.

DNA inhibitor for both vaccinia and herpes simplex viruses(2,3). In this case, again, there are differences in technic, host cells and media. It is worth noting that FDU has been reported as an inhibitor of thymidilic acid biosynthesis while IDU inhibits the incorporation of thymidine into DNA and is itself incorporated, resulting in so-called fraudulent DNA(5). Aminopterin also has been reported to influence DNA synthesis by interfering with thymidilic acid production(1). Perhaps interference with thymidilic acid biosynthesis is not an adequate mode of action for suppressing plaque formation in the system used here. Additional samples of FDU are being obtained so that the reason for the apparent discrepancy in the antiviral activity of this compound can be studied.§

§ The question has been raised as to whether halogenated deoxyuridines specifically inactivated DNA viruses rather than influenced their synthesis. The methods used by previously cited investigators (1,2) seem to have eliminated this possibility. Furthermore, studies in this laboratory with numerous virus inactivating substances have shown that the plaque-inhibition test is relatively insensitive to such materials, probably because they are applied sometime after the cells are infected.

TABLE II. Reversal by Thymidine of Plaque Inhibition Produced by 5-Halogenated Deoxyuridines.

	Plaque inhibition zone radii (mm)*			
	Vaccinia		Herpes simplex	
	5-BDU	5-IDU	5-BDU	5-IDU
Thymidine, 200 μg/disc				
Discs 5 mm apart	0	0, 0	0	0
" 30 " "	17	9, 12	10	12
Deoxyuridine, 200 μg/disc; discs side by side	17	17	17	17
Control	15, 20, 15	15, 17	15, 17	15, 17

* Diameter measurements were not used since thymidine reversal was most effective on that portion of the plaque-free zone lying between the 2 test discs. The radii reported were measured from center of halide deoxyuridine discs to edge of plaque inhibition zone in the direction of the thymidine or deoxyuridine discs. Data for controls are for the shortest radii. Fig. 1 illustrates the necessity for this method of measurement.

The data presented here are not to be considered quantitative since variations in the culture vessels result in differences in size of plaque-free zones. Nevertheless, there was indication that BDU was somewhat more active than IDU, which would agree with the *in vitro* results of Prusoff(6) in studying inhibition of DNA biosynthesis in mouse tumor cells. For example, zones of plaque inhibition produced by IDU contained at times a number of residual plaques while zones produced by BDU seemed to be truely plaque-free. This difference was most noticeable with herpes simplex virus.

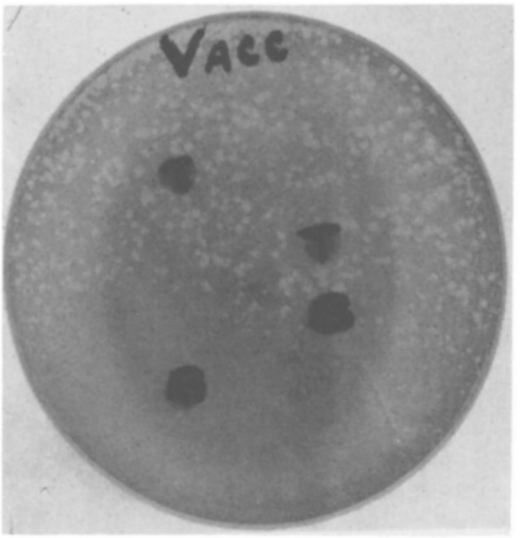

FIG. 1. Four day agar overlay culture containing plaques produced by vaccinia virus. B's cover discs with 20 μg of BDU, T's cover discs with 200 μg of thymidine. Note in the case of the pair of discs separated by 5 mm, to the right, that although plaques are well formed in the area of the discs some plaque suppression still occurs outside the area of thymidine diffusion (lower right of dish).

As might have been predicted from a previous report(1), when 200 μg of thymidine was placed in close proximity to 20 μg of IDU or BDU on a vaccinia or herpes simplex virus infected agar overlay culture, plaque inhibition was reversed (Table II, Fig. 1). When the distance between the thymidine disc and the halogen deoxyuridine discs was increased from 5 to 30 mm, plaque inhibition again was produced. There was some indication that even at 30 mm distance thymidine had some effect on IDU. This is an example of how media constituents could cause discrepancies in testing antiviral agents *in vitro*. If the media used here had contained significant levels of thymidine by some unusual circumstance, then plaque inhibition would not have been observed. It has been pointed out that FDU might be useful in determining nucleic acid content of various viruses(2). BDU and IDU, however, may be more reliable for this purpose.

Preliminary tests of IDU have been undertaken in vaccinia and herpes simplex infected eggs and animals, and there seemed to be some antiviral effect in eggs. The rapid breakdown of IDU in the animal body(6) could explain the apparent absence of antiviral activity in mouse tests. These *in vivo* studies will be reported later.

Summary. 5-Iodo- and 5-bromo-2′-deoxyuridine, when tested in the agar-diffusion

plaque-inhibition test, were found to inhibit plaque formation of DNA containing vaccinia and herpes simplex viruses but not RNA containing West Nile and Newcastle disease viruses. A number of other compounds that might be candidates for the disruption of normal nucleic acid biosynthesis, including aminopterin and 5-fluoro-2'-deoxyuridine, also were tested and found inactive in this test system. Thymidine readily reversed plaque inhibition by both 5-iodo- and 5-bromo-2'-deoxyuridine.

1. Smith, J. D., Freeman, G., Vogt, M., Dulbecco, R., *Virology,* 1960, v12, 185.

2. Salzman, N. P., *ibid.,* 1960, v10, 150.

3. Tamm, I., *Clin. Pharmacol. and Therap.,* 1960, v1, 777.

4. Herrmann, E. C., Jr., Gabliks, J., Engle, C., Perlman, P. L., PROC. SOC. EXP. BIOL. AND MED., 1960, v103, 134.

5. Welch, A. D., Prusoff, W. H., *Cancer Chemotherapy Repts.* 1960, Issue 6, 29.

6. Prusoff, W. H., *Cancer Research,* 1960, v20, 92.

Received February 20, 1961. P.S.E.B.M., 1961, v107.

Mammalian Degradation of (−)-Nicotine to 3-Pyridylacetic Acid and Other Compounds.* (26561)

HERBERT MCKENNIS, JR., EDWARD R. BOWMAN†, AND LENNOX B. TURNBULL
Dept. of Pharmacology, Medical College of Virginia, Richmond

Data obtained from administration of randomly labelled nicotine-C^{14} in the rat(1) and the dog(2) have indicated that over 90% of the administered radioactivity is excreted in the urine as metabolites and as unchanged nicotine. Metabolic studies(3-5) in the dog with non-isotopic (−)-nicotine, and the metabolic intermediate (−)-cotinine have led to the isolation and characterization of metabolites which account for approximately 30% of the administered dose, *viz.,* (−)-cotinine, (−)-desmethylcotinine, hydroxycotinine, (+) - γ - (3 - pyridyl) - γ - methylaminobutyric acid, and γ-(3-pyridyl)-β-oxo-N-methylbutyramide. In addition, experiments in the rat(6) with nicotine-C^{14}-methyl have clearly shown a metabolism of the methyl group of nicotine to respiratory carbon dioxide and urea.

The foregoing, which suggest a complexity in the metabolism of nicotine far in excess of estimates by other authors(7), have led to further investigation of the metabolism of randomly labelled (−)-nicotine-C^{14}. It has now been demonstrated that a sequence of metabolic reactions leads from (−)-nicotine to 3-pyridylacetic acid.

Materials and methods. Two male mongrel dogs, 11.7 and 13.8 kg under pentobarbital anesthesia received randomly labelled (−)-nicotine-C^{14}‡, 4.25 mg/kg, total radioactivity 1.07×10^6 CPM intravenously over an 8-hour period. The urine was collected from the bladder by an indwelling catheter during infusion and a subsequent 19-hour period. Radioactive determinations were made on infinitely thick samples which were dried on talc. Urine samples were processed by modifications of previously described procedures(3-5) and descending paper chromatograms were prepared on Whatman No. 1 paper as previously described(3-5).

Studies on the metabolism of (−)-cotinine were conducted on an adult male mongrel dog. (−)-Cotinine was administered orally (100 mg/kg). The animal received commercial pellets and water *ad libitum,* and urine (preserved with sodium fluoride) was collected as run-off from the metabolism cage.

* Supported by grants from the Tobacco Industry Research Committee and American Tobacco Co. Presented in part at the 14th Tobacco Chemists' Conf., Winston-Salem, N. C., Oct. 13, 1960.

† Public Health Research Fellow, Nat. Heart Inst.

‡ The authors are greatly indebted to Dr. L. J. Dewey and Dr. W. Stepka for this material which was extracted from *N. rustica* which was grown in an atmosphere of $C^{14}O_2$.