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INDIRECT EVIDENCE FOR THE PRESENCE OF RIBONUCLEIC ACID IN VESICULAR STOMATITIS VIRIONS *

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Concentration of 5 gamma g/ml or less of 5-fluorodeoxyuridine (FDU) and of 5-bromodeoxyridine (BDU) inhibited the growth of adenovirus type 5 and of vaccinia virus in ERK and chick embryo cells, provided that adequate time was allowed for the inhibitors to act. With the same proviso, FDU and BDU also inhibited the growth of ERK cells. Under similar conditions of cultivation, concentrations of up to 100 gamma g/ml of FDU and of BDU had no effect on the growth of either the New Jersey or the Indiana serotype of vesicular stomatitis virus (VSV) in either ERK or chick embryo cells. It is concluded that virus particles of both serotypes of VSV contain RNA rather than DNA.

INTRODUCTION

It is difficult at present to relate the two serotpyes of vesicular stomatitis virus (VSV) to other viruses. These serotypes are themselves antigenically unrelated to one another, although biologically similar. There is evidence that arthropod vectors may be involved in the natural spread of vesicular stomatitis virus, and Mussgay and Suarez (1962) have demonstrated directly that VSV can grow in *Aedes aegypti* mosquitoes. But despite its convenience, the use of habitat as a primary criterion for classification of animal viruses presents a number of anomalies (Cooper, 1961a). As an example of such an

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anomaly, the structure of VSV particles (Reczko, 1960; Howatson and Whitmore, 1962) does not resemble that of other arboviruses, and in fact may be unusual or even unique among the smaller viruses that infect vertebrates.

It has been suggested (Cooper, 1961a) that for certain purposes there is merit in a formal classification of animal viruses based on the type of nucleic acid contained in the virus particle as a primary criterion. The work reported here was done with this in mind and uses the suggestion of Salzman (1960) that inhibitors of DNA synthesis can be used to differentiate between viruses containing RNA and those containing DNA.

This paper demonstrates the conditions under which two inhibitors of DNA synthesis, 5-fluorodeoxyuridine (FDU) and 5-bromodeoxyuridine (BDU), were effective in the virus growth systems employed. Using these conditions, it is shown that FDU and BDU have no effect on the growth of either serotype of VSV.

MATERIALS AND METHODS

Samples of FDU and BDU were obtained through the courtesy of Dr. Ronald Ross, Public Health Service, Department of Health, Education, and Welfare, Bethesda, Maryland.

Vesicular stomatitis virus, obtained from the Research Institute, Pirbright, Surrey, England, was grown and assayed in 16-hour chick embryo cell monolayers. The plaque method described by Cooper (1957a) was used, with the difference that the liquid medium was CSV.6 (Cooper *et al.*, 1959), and the agar overlay medium was that described by Cooper (1961b). Titres are expressed as plaque-forming units per millilitre (PFU/ml).

Poliovirus was assayed by the agar cellsuspension plaque method (Cooper, 1961b), in ERK cells grown as described by Cooper *et al.* (1959). Titres are expressed as PFU/ml.

Adenovirus type 5, obtained from the National Institute of Medical Research, Mill Hill, London, was grown in ERK cells in CSV.6, and assayed in the same cells by the following end-dilution method. Serial tenfold dilutions of a preparation of unknown titre were added in 0.1-ml amounts to 16-hour tube culture (8 tubes per dilution), each tube containing 2.4×10^4 ERK cells in 2 ml CSV.6. Cytopathic effect (CPE) was recorded after 8 days at 36°; the dilution factor which produced detectable CPE in half of the tubes at this time was interpolated, and the titres (which equal the reciprocal of the dilution factor) are expressed as tissue culture infectious doses per millitre.

Vaccinia virus was obtained both from the Lister Institute, Elstree, Hertfordshire, England, and from the Pasteur Institute, Iran. Its infectivity

was assayed as for adenovirus except that CPE was recorded after 4 days at 36°.

RESULTS

Effect of FDU on Growth of Adenovirus Type 5 in ERK Cells and of Vaccinia Virus in ERK and Chick Embryo Cells

The efficacy of FDU in inhibiting DNA synthesis in the systems to be used to test VSV was first checked by its effect on the growth of two viruses known to contain DNA (vaccinia virus, Wyatt and Cohen, 1953; adenovirus type 5, Allison and Burke, 1962). Table 1 shows that, when the inoculum was very small, FDU inhibited the CPE of adenovirus in ERK cells, and of vaccinia virus in ERK and chick embryo cells. The inhibition was complete down to 3 gamma g/ml, and partial at some lower concentrations. Production of infectivity was strongly inhibited by 3 gamma g FDU/ml. However, FDU failed to protect ERK cells against somewhat larger inocula, a result suggesting that in these cases FDU had not been present sufficiently in advance of the final cycle of virus growth.

Effect of FDU on the Growth of ERK Cells

The experiments described in the preceding paragraph show that FDU was not always able to inhibit DNA synthesis in the cell system sued. In order to be sure that FDU was exerting its full effect, ERK cells were henceforth incubated with FDU before infection until the cells had ceased to divide; it is presumed that DNA synthesis will then also have ceased. The following experiment indicated the amount of preincubation with FDU required to stop ERK cell division.

Twelve replicate ERK cell cultures were set up in Pyrex bottles; 6 of the cultures contained 10 gamma g of FDU per milliter. The cultures were incubated at 36° without change of medium at any stage, and at intervals one control and one FDU-containing culture were trypsinized and the cells were counted. Figure 1 shows the total recoveries of cells in both sets of cultures; the inhibition of the growth of ERK cells was not complete until 4—5 days after the addition of FDU. A similar experiment with 50 gamma g/ml of FDU indicated that inhibition by this higher concentration was not complete until 3 days after the addition of FDU.

Effect of FDU on the Growth of VSV and Poliovirus in ERK and Chick Embryo Cells

Cells in which the growth of VSV was to be tested were preincubated

with concentrations of FDU (50 and 100 gamma g/ml) and for a time (7 days) in excess of time and concentration shown to be sufficient for inhibition of the growth of ERK cells (see Fig. 1) and of DNA-containing viruses (see Table 1). Poliovirus was included in the test series as a virus known to contain RNA (Schwerdt and Schaffer, 1955), and was treated in the same way as was VSV. The results of the tests of virus growth were measured in terms of production of infectivity and of CPE. However, since several cycles of vir s growth must occur if the presence of CPE is to indicate synthesis of

TABLE 1

EFFECT OF FDU ON THE GROWTH OF VACCINIA VIRUS AND OF ADENOVIRUS TYPE 5[a]

Virus	Cell type	Inoculum (infectious doses per tube)	FDU (μg/ml)							
			0	0.1	0.3	1.0	3.0	10	30	
Adeno	ERK	1000	3[b]	3[b] —	—	3[b]	3[b] —	3[b]	3[b]	
		100	3	3 —	—	3	2	1	1 0	—
		10	3	2 $>10^4$[c]	—	1	0 10^1[c]	0	0	$<10^1$
		1	2	1	—	0	0 —	0	0	—
		0	0	0 —	—	0	0 —	0	0	—
Vaccinia	ERK	100	3[d] —	3[d]	3[d]	3[d]	3[d] —	3[d]	3[d]	—
		10	3	3	3	3	3 —	3	3	—
		1	2 10^2[c]	2	1	0, 1	0 10^1[c]	0	0	$<10^1$
		0	0	0	0	0	0 —	0	0	—
Vaccinia	Chick embryo	10^5	1[e] —	—	1[e]	0[e]	0[e] —	0[e]	0[e]	
		10^4	10^3[c]	—	1	0	0 $<10^1$[c]	0	0	$<10^1$
		10^4 to 10	1 —	—	1	0	0 —	0	0	—
		0	0 —	—	0	0	0 —	0	0	

[a] One series of tubes received 2.4×10^5 ERK cells in 2 ml CSV 6, and another series received 5×10^6 chick embryo cells in 1 ml CSV.6. FDU was added to the indicated concentrations, and the tubes were incubated at 36°. After 5 hours, 0.1 ml of CSV.6 containing the indicated inocula were added to the tubes. Incubation was continued, and the tubes were examined daily for CPE. Control cells in absence of virus were morphologically normal at the end of the experiment, although those in presence of 10 and 30 μg/ml of FDU were slightly changed. Relative CPE is expressed as 0, 1, 2, or 3, indicating, respectively, zero, up to 10%, 10–90%, or all cells showing CPE. All tubes were duplicated, and both readings of CPE are given if duplicates differed.

[b] Relative CPE after 8 days at 36°.

[c] Infective titre from an end-dilution method, of material harvested at the time for which CPE is reported.

[d] Relative CPE after 4 days at 36°.

[e] Relative CPE after 7 days at 36°.

viral nucleic acid, very small inocula of VSV and poliovirus, were used. This additionally provided a further period of up to 24 hours in the presence of FDU before the final cycle of virus growth occurred.

Table 2 indicates that the infectivity produced by either serotype of

VSV in chick embryo or ERK cells was independent of the presence of FDU. Poliovirus growth was also unaffected.

CPE was complete in all infected cultures, and there was no significant difference in the rate of appearance of CPE in presence or absence of FDU in any particular cell-virus combination of the experiment described in Table 2. The size of the inoculum, within the limits used, also had no detectable effect on production of infectivity or of CPE. Uninfected control cells showed no cytopathic effect resembling that of virus, although FDU had produced slight morphological changes in the cells by the end of the experiment.

Effect of BDU on Growth of VSV in Chick Embryo and ERK Cells

As a control of the efficacy of BDU in chick embryo cells, two 1-liter flasks each containing a monolayer of 10^8 chick embryo cells were infected with about 200 tissue cultures infective doses of vaccinia virus in 2 ml CSV.6.

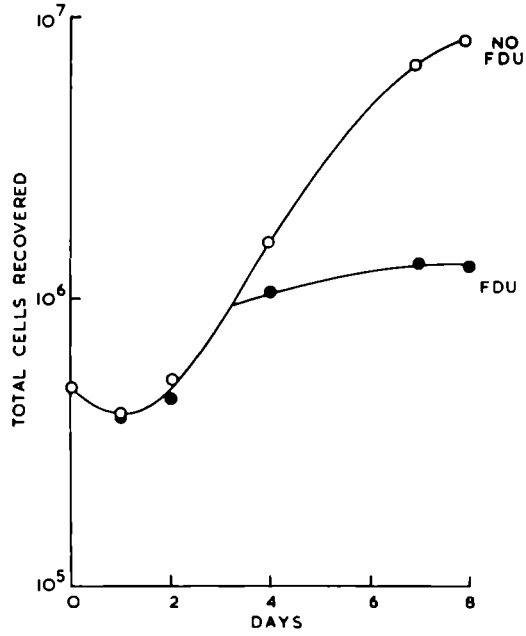

Fig. 1. Effect of FDU on the growth of ERK cells. Two series of 6 cultures, each containing 5×10^5 ERK cells in 10 ml CSV.6, were placed in Pyrex bottles at 36° at zero time. One series contained 10 gamma g FDU/ml. One culture from each series was trypsinized at the times indicated on the abscissa, and the cells were counted. Les than 7% of the inoculum and of all harvested cells absorbed stain from trypan blue solution (2 mg/ml).

After 1 hour's adsorption, the cultures received 100 ml of medium CSV.6, and BDU was added to one culture to a concentration of 5 gamma g/ml. Incubation proceeded for 4 days at 36°, when the culture without BDU contained fifty to one hundred macroscopically visible plaquelike areas of morphologically altered cells, which became confluent by 6 days. The culture in presence of BDU remained morphologically unchanged. Infectivity tests were not performed.

In order to test the effect of BDU on the growth of VSV in chick embryo cells, three series of tubes containing 3×10^3 chick embryo cells in 1 ml of medium CSV.6 were incubated for 7 days in absence of BDU or in presence of 50 or 100 gamma g BDU/ml, respectively. Tubes of each series were then infected by the addition of 0.1 ml medium containing eitheer (a) 1, 10, or 100 PFU of the Indiana serotype of VSV, or (b) 1, 10, or 100 PFU of the New Jersey serotype of VSV, or (c) no virus; each tube was duplicated.

Whether in presence or absence of BDU, complete viral cytopathic effect was present in all infected cultures after a further 2 days at 36°; at this time cultures were harvested and duplicate tubes pooled for assay of infectivity. Uninfected control cells showed no cytopathic effect resembling that of virus,

TABLE 2

THE EFFECT OF FDU ON THE GROWTH OF THE INDIANA AND NEW JERSEY SEROTYPES OF VSV AND OF POLIOVIRUS IN ERK[a] AND CHICK EMBRYO[b] CELLS

Virus inoculum[c]	Cell type	FDU concentration, μg/ml		
		0	50	100
Poliovirus	ERK	50 to 80 × 10^5 [d]	5 to 65 × 10^5	9 to 48 × 10^5
VSV (New Jersey)	Chick embryo	10 to 200 × 10^5	5 to 10 × 10^5	15 to 32 × 10^5
VSV (New Jersey)	ERK	10^5 to 10^6	10^5 to 10^6	10^5 to 10^6
VSV (Indiana)	Chick embryo	15 to 35 × 10^6	13 to 44 × 10^6	2 to 20 × 10^6
VSV (Indiana)	ERK	10^5 to 10^6	10^5 to 10^6	10^5 to 10^6

[a] Two series of tubes containing 8 × 10^4 ERK cells in 1 ml of CSV.6 were incubated at 36° for 7 days in presence of 50 or 100 μg FDU per milliliter, respectively; an identical control series containing no FDU was set up on the sixth day.

[b] Three series of tubes each containing 3 × 10^5 chick embryo cells in 2 ml CSV.6 were incubated at 36° for 7 days in presence of zero, 50, or 100 μg FDU per milliliter, respectively.

[c] On the seventh day, all tubes received 0.1 ml of CSV.6 containing either zero, 1, 10, 100, or 1000 PFU. All samples were duplicated. Tubes were examined daily for CPE, and harvested 3 days (poliovirus in ERK cells, and VSV in chick embryo cells) or 3 to 8 days (VSV in ERK cells) after infection, when viral CPE was complete in all infected cultures. Duplicate tubes were pooled for assay of infectivity.

[d] Values are range of yields produced, in PFU/ml.

although BDU had produced slight morphological changes in the cells by the end of the experiment.

No marked effect of BDU on the production of VSV infectivity was

found. Control cultures infected with the Indiana serotype produced 2 to 5.5×10^6 PFU/ml, whereas 50 and 100 gamma BDU/ml, respectively, permitted production of 1.5 to 2.75×10^6 and 0.65 to 1.6×10^6 PFU/ml of this virus. All cultures of the New Jersey serotype produced between 0.84 and 2.5×10^6 PFU/ml, whether in presence or absence or absence of BDU.

In order to test the effect of BDU on the growth of VSV in ERK cells, cultures of ERK cells were preincubated in presence of 100 gamma g BDU/ml until DNA synthesis could safely be presumed to be inhibited. The growth of these culturese is compared in Fig. 2 with the growth of cultures containing no BDU. Growth of ERK cells had ceased in presence of BDU by the sixth day, but incubation was continued until the tenth day, whon the BDU containing cultures were infected in duplicate with about 500 PFU of either the New Jersey or the Indiana serotype of VSV; two cultures were left uninfected. As controls, fresh cultures had been set up in the absence of BDU

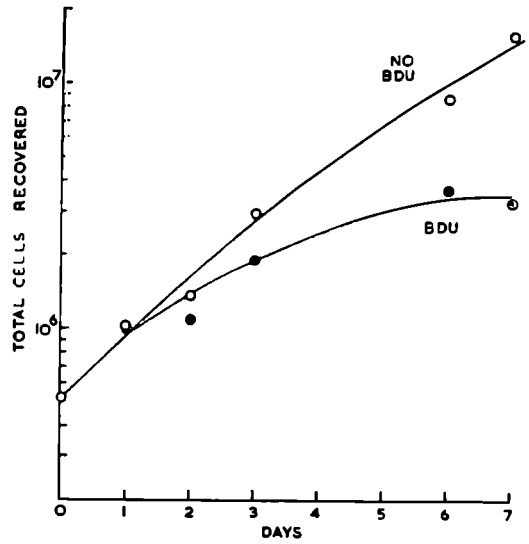

Fig. 2. Effect of BDU on the growth of ERK cells. Two series of 12 cultures, each containing 5×10^5 ERK cells in 5 ml CSV.6, were placed in Pyrex bottles at 36° at zero time. One series contained 100 gamma g BDU/ml. One culture from each series was trypsinized at the times indicated on the abscissa, and the cells were counted. Less than 5% of the inoculum and of all harvested cells absorbed stain from trypan blue solution (2 mg/ml). After cell growth had ceased in the cultures containing BDU, these cultures were used for the growth of both serotypes of VSV (see text).

on the seventh day, and were infected in the same manner and at the same time as the BDU-containing cultures. The number of cells per culture was about the same in control and in infected cultures. Incubation continued at 36° for 4 days after infection, by which time appreciable degeneration was evident in the noninfected cultures containing BDU. CPE was then complete in all cultures infected with the Indiana serotype, and partial in all cultures infected with the New Jersey serotype. The extent and rate of onset of CPE were identical in cultures with or without BDU. On the fourth day after infection, the cultures were harvested and duplicates were pooled for assay of infectivity. Preincubation in presence of BDU produced no significant effect on the yields of infectivity: the Indiana serotype yielded 1.3×10^6 PFU/ml in absence of and 1.0×10^6 PFU/ml in presence of BDU, and the New Jersey serotype yielded 10^4 PFU/ml in absence and 7.5×10^3 PFU/ml in presence of BDU.

DISCUSSION

The chemical structure of FDU and BDU lead to the expectation that both analogs will interfere with DNA synthesis, and this expectation is borne out in practice (Cohen *et al.,* 1958; Simon, 1961; Wilson and Dinning, 1961). Therefore growth of DNA-containing viruses should also be inhibited by these analogs, and this also is substantiated (Salzman, 1960; Simon, 1961). The effect on growth of RNA-containing viruses is less easy to predict, but Salzman (1960) has reported that FDU has no effect on the growth of poliovirus whereas vaccinia virus is markedly inhibited. Simon (1961) has used the lack of effect of aminopterin, 5-fluorouracil, and BDU on the growth of known RNA-containing viruses (poliovirus and Newcastle disease virus) to conclude that synthesis of viral RNA is, in these instances at least, independent of cellular DNA or DNA synthesis. Thus, provided that the analogs can be shown to be effective in the system used for growing the virus, there seems to be good reason for accepting the lack of effect of FDU and BDU on the growth of a particular virus as indicating that the infective particles formed contain no DNA, and therefore presumably contain RNA. However, in seeking to demonstrate such a negative result, it is desirable to use doses considerably in excess of the smallest effective dose. It is also desirable to use both FDU and BDU since they probably act on different stages of DNA synthesis (Cohen *et al.,* 1958; Simon, 1961), and to use more than one cell type.

In the present work, it was necessary to conduct a number of control experiments to ensure that conditions were used in which FDU and BDU

were fully effective. These conditions involved extensive preincubation with high concentrations of the analogs and yielded data which all indicate that virions of both the New Jersey and Indiana serotypes of vesicular stomatitis virus contain RNA rather than DNA. This conclusion is supported by the earlier investigations of Cooper (1957b) and of Turco (1959), which indicate marked changes in RNA metabolism of VSV-infected cells with little or no DNA synthesis or breakdown. A more direct support also comes from the experiments of Cooper (1957b), in which small amounts of particulate RNA-containing material were liberated from infected cells at the same time as the infectivity and before cell breakdown became apparent; these particles sedimented in the ultracentrifuge and adsorbed to infusorial earth in a fashion similar to the infectivity.

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REFERENCES

ALLISON, A. C., and BURKE, D. C. (1962). The nucleic acid content of viruses. *J. Gen. Microbiol.* 27, 181-194.

COHEN, S. S., FLAKS, J. G., BARNER, H.D., LOEB, M. R., and LICHTENSTEIN, J. (1958). The mode of action of 5-fluorouracil and its derivatives. *Proc. Natl. Acad. Sci. U. S.* 44, 1004-1012.

COOPER, P. D. (1957a). Some characteristics of vesicular stomatitis virus growth-curves in tissue culture. *J. Gen. Microbiol.* 17, 327-334.

COOPER, P. D. (1957b). Paths of phosphate transfer in normal chick embryo cells and in cells infected with vesicular stomatitis virus. *J. Gen. Microbiol.* 17, 335-352.

COOPER, P.D. (1961a). A chemical basis for the classification of animal viruses. *Nature* 190, 302-305.

COOPER, P. D. (1961b). An improved agar cell-suspension plaque assay for poliovirus : some factors affecting efficiency of plating. *Virology* 13, 153-157.

COOPER, P. D. WILSON, J. N., and BURT, A. M. (1959). The bulk growth of animal cells in continuous suspension culture. *J. Gen. Microbiol.* 21, 702-720.

HOWATSON, A. F., and WHITMORE, G. F. (1962). The development and structure of vesicular stomatitis virus. *Virology* 16, 466-478.

MUSSGAY, M., and SUAREZ, O. (1962). Multiplication of vesicular stomatitis virus in *Aedes aegypti* (L.) mosquitoes. *Virology* 17, 202-204.

RECZKO, E. (1960). Elektronenmikroskopische Untersuchungen am Virus der stomatitis vesicularis. *Arch. ges. Virusforsch.* 10, 588-605.

SALZMAN, N.P. (1960). The rate of formation of vaccinia deoxyribonucleic acid and vaccinia virus. *Virology* 10, 150-152.

SCHWERDT, C. E., and SCHAFFER, F. L. (1955). Some physical and chemical properties of purified poliomyelitis virus preparations. *Ann N. Y. Acad. Sci.* 61, 740-750.

SIMON, E. H. (1961). Evidence for the nonparticipation of DNA in viral RNA synthsis. *Virology* 13, 105-118.

TURCO, G. (1959). Changes in cellular ribonucleic acid during growth of vesicular stomatitis virus in chick cell culture. *J. Gen. Microbiol.* 21, 510-518.

WILLSON, J. C., and DINNING, J. S. (1961). Effects of 5-Br-deoxyuridine and 5-F-deoxyuridine on nucleic acid metabolism by rabbit bone marrow cells. *Proc. Soc. Exptl. Biol. Med.* 108, 366-369.

WYATT, G.R., and COHEN, S.S. (1953). The bases of the nucleic acids of some bacterial and animal viruses; the occurrence of 5-hydroxymethylcytosine. *Biochem. J.* 55, 774-782.