tion" with and on the state (latent?) of such agent.

Four of our 5 lines became more resistant than primary cultures to wild-type polioviruses; the relative resistance of the later passage levels of the π line was particularly marked. The fifth line (κ) remained highly susceptible; using eop as criterion, it was consistently even slightly more susceptible than primary cultures to the type 3 poliovirus used. These results are rather similar to those of Hull, Cherry, and Tritch(7), who found their 2 lines, when tested uncloned, to be slightly more resistant than primary cultures to type 1 poliovirus, and to the results of Takaoka *et al*(8), who found their cynomolgus line to be clearly less sensitive than primary cultures to type 1 polioviruses; both of these groups of workers used end point titrations in tube cultures under liquid medium.

Some of the 9 mutants described herein may be identical with each other: Insofar as tested, the Brunhilde m_α and m_ϵ mutants did not differ significantly from each other; nor did the Mabie r_{pr}, r_η, and r_π mutants. Because of the relative ease of distinguishing them from wild type, the 3 *m* mutants and the Mabie r_κ mutant should be appropriate for certain studies requiring genetically marked polioviruses.

Summary. Five lines of rhesus monkey kidney cells were established. Four of the 5 became more resistant than primary rhesus kidney cultures to wild-type polioviruses; the sensitivity of the remaining line (κ) to wild-type polioviruses was roughly equal to that of primary cultures. All 5 lines were susceptible to poliovirus RNA, though some were more susceptible than others. Passages of polioviruses on the lines selected 3 *minute-plaque* and 5 *rapid* poliovirus mutants.

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Multiplication of Canine Hepatitis Virus in the Presence of 5-Fluorodeoxyuridine.* (29092)

J. E. MOULTON AND L. M. FRAZIER (Introduced by Clyde Stormont)
Department of Pathology, School of Veterinary Medicine, University of California, Davis

In a recent study(1) canine hepatitis (CH) virus was observed to increase in cultures of dog kidney (DK) cells when the cultures had been treated with 5-fluorodeoxyuridine (FUDR) at concentrations that inhibit other DNA viruses. This lack of inhibitory effect of FUDR upon CH virus was observed in cultures incubated 30 hours or longer but not in cultures incubated 18 to 24 hours. A possible explanation for this lack of inhibition is presented in this report. Further studies have been made of cultures with and without FUDR; changes in cell morphology, DNA, and virus were determined.

Materials and methods. The procedure

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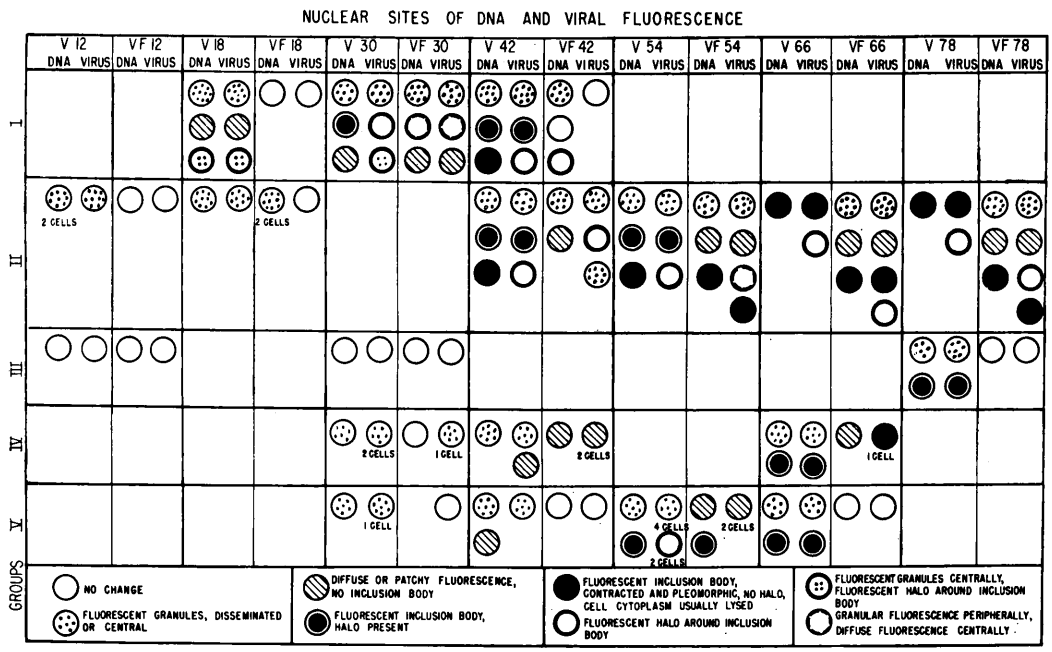

FIG. 1. Nuclear changes at varying periods of infection with acridine orange (DNA) and fluorescent antibody (virus) staining. The symbols V and VF refer to virus-infected and virus-infected, FUDR-inhibited respectively. The hours after infection are indicated by the numbers. Circles in the chart represent nuclei; nuclear changes are represented by symbols at bottom of chart. Groups are listed at left. Number of cells affected in a culture are entered when few in number.

used in preparing secondary cultures of DK cells on coverslips in Leighton tubes has been described(2). Cultures for virus titrations were grown in prescription bottles, removed, packed by centrifugation, then resuspended in one ml of spent medium that had been centrifuged to remove dead cells. Cell suspensions were frozen and thawed 10 times to release intracellular virus and titrated by the tube dilution method(2). The titers of virus used to infect the cultures were as follows: 10^5TCID$_{50}$ in Group I, 10^4TCID$_{50}$ in Groups II and III, and 10^2TCID$_{50}$ in Groups IV and V. The inoculated virus was left in the cultures for the entire incubation period. At 18 hours before infection, 10^{-5} M (final concentration) FUDR was added to part of the cultures in Group I, and 10^{-4} M FUDR in Groups II and III. In one group, 10^{-3} M FUDR was used, but was found toxic to the cells. Non-infected cultures, with and without FUDR, were included in each group as controls. Cultures with virus alone and with virus plus FUDR (henceforth abbreviated V and VF respectively), and controls were removed after 6, 12, 18, 30, and 42 hours in Group I; 6, 12, 18, 42, 54, 66, and 78 hours in Group II; 12, 30, and 78 hours in Group III; and 30, 42, and 66 hours in Groups IV and V.

At each time interval cultures on cover slips were fixed in acetone for fluorescent antibody (FA) staining and in Carnoy's acetic-alcohol for staining by Feulgen and acridine orange (AO) methods. Both direct and indirect FA techniques were used(3). Most of the work was done using the indirect method with rhodamine added(4) to the conjugate† to mask non-specific fluorescence. The staining methods for Feulgen and AO have been described(5,6).

Examinations were made of 56 cultures stained with FA, 15 cultures stained by the Feulgen method, and 56 cultures stained with AO. FA and AO preparations were exam-

† Rabbit anti-canine gamma globulin labelled with fluorescein isothiocyanate, prepared by Dr. J. H. Hillman, Antibodies, Inc., Davis, Calif.

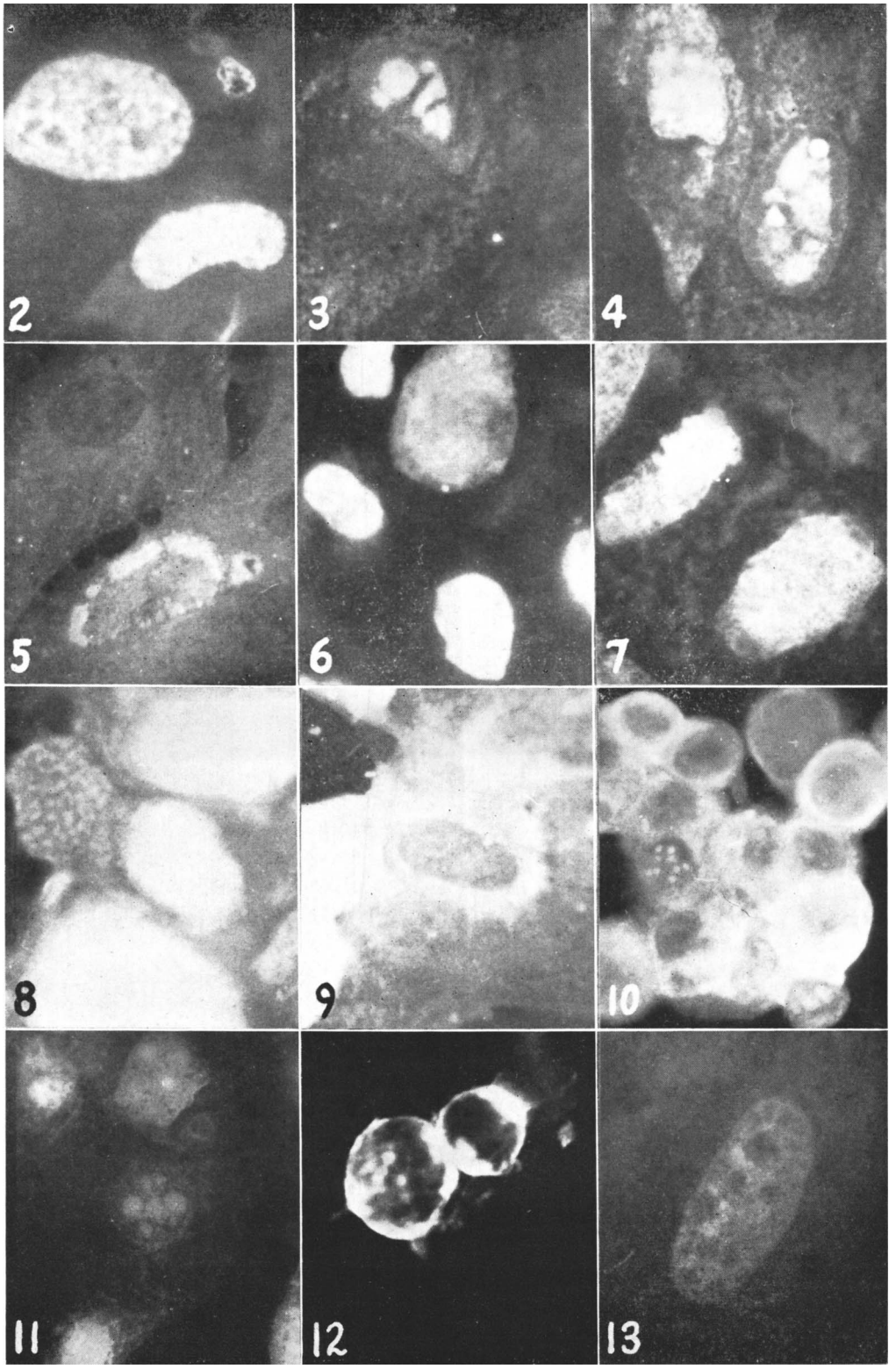
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FIG. 2. Two nuclei with closely packed, DNA-staining granules in an 18-hr, virus-infected culture. Acridine orange (× 600).

FIG. 3. Nucleus with large, DNA-staining granules in culture 18 hr post-infection. Granules are collected in center of nucleus, prior to merging into a single inclusion body. Acridine orange (× 600).

FIG. 4. Two nuclei in 18-hr, infected culture. DNA granules in one nucleus have almost consolidated into an inclusion body. Observe halo formation and 2 nucleoli (small, round, white objects) that have moved toward nuclear margin (margination). Granules are disseminated throughout the nucleus in other cell. Acridine orange (× 600).

FIG. 5. One cell has granules with specific immunofluorescence for viral antigen at periphery of nucleus. Some immunofluorescence in center of nucleus also. Compare with other cell which is normal. Fluorescent antibody (× 600).

FIG. 6. Nuclei with inclusion bodies, 42 hr after infection. Inclusion bodies stain brightly for DNA and are located in contracted cells. Observe also, single nucleus with diffuse DNA staining. Acridine orange (× 600).

FIG. 7. Diffuse accumulations of DNA in nuclei of 42-hr, infected culture with added FUDR. Observe irregular distribution of DNA staining material. Acridine orange (× 600).

FIG. 8. DNA-staining inclusion bodies lacking definite halos in 54-hr, infected culture. Observe brightness of staining. One small nucleus has DNA granules. Acridine orange (× 600).

FIG. 9. Immunofluorescence at periphery of an inclusion body 54 hr after infection. Fluorescence is not limited to nucleus, but extends into cytoplasm (staining artifact?). Fluorescent antibody (× 600).

FIG. 10. Immunofluorescence at peripheries of contracted inclusion bodies in cells with lysed cytoplasm in culture infected for 66 hr. One nucleus has granular fluorescence. Fluorescent antibody (× 600).

FIG. 11. Observe pale, diffuse and granular DNA staining deposits in nuclei of culture infected for 66 hr and inhibited with FUDR. Acridine orange (× 500).

FIG. 12. Bright peripheral immunofluorescence in 2 nuclei with inclusion bodies in culture 78 hr post-infection. Fluorescent antibody (× 500).

FIG. 13. Nucleus of non-infected cell in control culture with added FUDR. Note multiple clear areas in nucleus. Similar change was noted in small cells in infected, inhibited cultures. Acridine orange (× 600).

ined in a fluorescence microscope; Feulgen preparations were examined by microspectrophotometry. In the latter, light absorbed by the Feulgen method was measured and used as a basis for computing the quantity of DNA(5). A monochromator‡ was used to isolate light at a wave length of 546 mμ. All measurements were made at 582 × magnification under oil immersion. A total of 50 nuclei selected at random were measured per culture. Appropriate controls were included with each stain.

Results. Acridine orange and fluorescent antibody. The time sequence of AO and FA changes is illustrated in Fig. 1. Normal cells stained with AO had green fluorescence for DNA and red fluorescence for RNA. Viral immunofluorescence was green.

Group I. After 18 hours the virus-infected culture (virus control) had DNA- and immuno-fluorescing granules in many nuclei (Fig. 2, 3, 4). Small granules were disseminated throughout the nucleus; large granules tended to collect in the center or at the margin (Fig. 5). There were no changes in the FUDR-inhibited culture. After 30 hours of infection inclusion bodies appeared in the nuclei. A typical inclusion body had a halo with marginated chromatin and nucleoli; the cytoplasm of a cell with an inclusion body was vacuolated, dendritic, or contracted. The inclusion body usually had diffuse fluorescence for DNA and peripheral fluorescence for virus. The 30-hour inhibited culture had granular and diffuse fluorescence of nuclei. After 42 and 66 hours of infection most of the cells in the virus controls contained inclusion bodies and only a few had nuclear granules (Fig. 6). The inhibited cultures had nuclear granules or diffuse fluorescence (Fig. 7).

Group II. After 12 and 18 hours nuclear granule fluorescence was observed in the virus controls. Two nuclei in the 18-hour inhibited culture had granules. Inclusion bodies were found 42 hours after inoculation in the control culture; nuclear granules and diffuse nuclear fluorescence were found in the inhibited culture. The few cells that remained in the virus control cultures after 54 and 66

‡ Continuous running filter monochromator, Carl Zeiss, Inc., N. Y.

TABLE I. Virus Titers and FUDR Concentrations in Cultures Used in Microspectrophotometry.

Group No.	Treatment (V = virus, VF = virus + FUDR)	Hr post-infection	Inoculating virus titer* ($TCID_{50}$, log_{10})	Concentration of FUDR (molar) †	Final virus titer‡ ($TCID_{50}$, log_{10})
I	V	42	5		10
	VF	42	5	10^{-5}	10
	V	66	5		
	VF	66	5	10^{-5}	
III	V	78	3		8.5
	VF	78	3	10^{-4}	4.4
IV	V	66	2		5.2
	VF	66	2	10^{-4}	3.6
V	V	66	2		7.3
	VF	66	2	10^{-4}	4.2

* Virus added at zero hour.
† FUDR added 18 hr before infection.
‡ Titrations made on cells plus culture medium.

hours of incubation usually contained pleomorphic inclusion bodies with diffuse or marginal immunofluorescence (Figs. 8-10). Nuclear granules were found in the inhibited cultures (Fig. 11). After 78 hours of infection inclusion bodies predominated in both inhibited and non-inhibited cultures (Fig. 12).

Group III. The first change in the virus control was found after 78 hours of infection. At this stage most of the affected cells had nuclear granules or inclusion bodies. There was no change in the inhibited culture.

Groups IV and V. The principal changes 30 and 42 hours after inoculation in inhibited and non-inhibited cultures were nuclear granule formation and diffuse fluorescence. Only a few cells were involved in each culture. Inclusion bodies were observed commonly after 54-66 hours in the virus controls; only a few cells had inclusion bodies in the inhibited cultures.

Controls. Non-infected cells in cultures with FUDR had thickened chromatin threads and decreased DNA and RNA fluorescence. Some of the cells had multiple, unstained foci in the nuclei (Fig. 13). Similar changes were found in infected cultures with FUDR.

DNA and virus. Increases in virus titers from time of inoculation and concentrations of FUDR in cultures stained by the Feulgen method and examined by microspectrophotometry are given in Table I. Comparisons of nuclear DNA in inhibited, non-inhibited, and control cultures at varying periods of infection are illustrated in Fig. 14-17. Control values are shown in Fig. 16-17. In Group I which received $10^5 TCID_{50}$ of virus, the mean level of DNA was increased in the virus control cultures after 42 and 66 hours of infection (Fig. 14). The inhibited cultures had no change in the mean level of DNA but had significant increase of DNA in one cell after 66 hours of infection. The final titers of virus were increased in both inhibited and non-inhibited cultures (Table I). In Group III which had $10^3 TCID_{50}$ of virus, the mean values of DNA were normal in inhibited and non-inhibited cultures after 78 hours of infection (Fig. 15). The final titers of virus were increased in these cultures but lower than in Group I (Table I). In Groups IV and V, which received $10^2 TCID_{50}$ of virus, there were slight increases in mean levels of DNA, and definite increases in a few cells in both inhibited and non-inhibited cultures after 66 hours of infection (Fig. 16, 17). The final titers of virus were increased but less than in other groups (Table I).

Discussion. These results indicate that CH virus multiplies in the presence of the DNA inhibitor, FUDR in concentrations of FUDR known to inhibit growth of other DNA viruses. Possible explanations for this are: 1) insufficient amount of FUDR, 2) degradation of FUDR during prolonged incubation, 3)

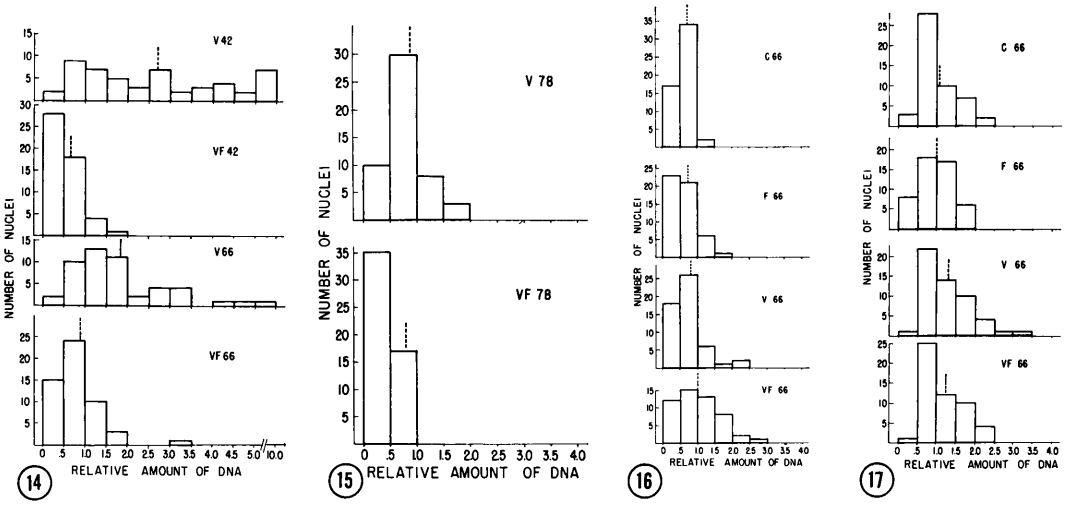

FIG. 14. DNA (in arbitrary units) after 42 and 66 hr of infection in FUDR-inhibited (VF) and non-inhibited (V) cultures in Group I. The mean values of DNA (dotted lines) may be compared on the graphs. Control values of DNA are shown in Fig. 16 and 17. Observe in Group I the large number of nuclei with increased DNA and high mean values in the non-inhibited cultures after 42 and 66 hr of incubation. In contrast, mean values in the inhibited cultures are within normal range although a significant increase in DNA is found in one cell in VF 66.

FIG. 15. Mean values of DNA in the V 78 and VF 78 cultures of Group III are similar although there are more nuclei with higher values in V 78.

FIG. 16. In Group IV observe control values of DNA after 66 hr of incubation in normal cells (C) and FUDR-treated cells (F). In the infected cultures, inhibited or not, there is a slight increase in mean values of DNA and definite increases in a few cells.

FIG. 17. Observe control values of DNA after 66 hr in Group V. Mean values are slightly increased in infected cultures and increases are seen in a few cells in V 66.

development of an alternate pathway of nucleic acid synthesis, 4) utilization of chromosomal DNA for virus synthesis, or 5) synthesis of DNA in cells that have become resistant to FUDR.

The amounts of FUDR used in the present study should have been sufficient for inhibition of virus for they were within the range proved effective in blocking other DNA viruses(9-13). Furthermore, these concentrations were effective in preventing multiplication of CH virus in shorter periods of incubation(1). The possibility that FUDR became degraded during prolonged incubation was considered by Flanagan and Ginsberg (13) in explaining the escape of types 4 and 5 adenovirus from inhibition. They observed inhibition of viral synthesis in cultures incubated for short periods, but lack of inhibition in cultures incubated for long periods. They concluded that the escape from inhibition was due to degradation of FUDR during prolonged incubation. This does not explain the escape of CH virus from inhibition though, for in a previous study(1) FUDR was added to cultures with and without CH virus and incubated for 18 hours at 37°C, and then the culture media were added separately to new cultures that were infected with virus and incubated for 24 hours. The entire period of incubation for FUDR was 42 hours. When titrated after 24 hours, virus synthesis was inhibited in these cultures as well as in cultures containing fresh FUDR. If FUDR had been denatured the titers would have been as high as those in non-inhibited cultures. If an alternate pathway of viral DNA synthesis developed, increasing the concentration of FUDR would have no effect on inhibition of the virus. However, both adenovirus(13) and CH virus(1) were inhibited in prolonged cultures when the concentrations of FUDR were increased. It was previously suggested that perhaps chromosomal DNA was used for virus synthesis in inhibited cultures. This was based

on an observation with fluorescent antibody of immunofluorescence in the chromosomes of inhibited cells. However, though a wide variety of fluorescent antibody techniques were employed in the present study this finding could not be confirmed. Furthermore, it would seem unlikely that chromosomal DNA, probably with a different nucleotide base ratio, could be used for virus synthesis.

The observed failure of FUDR to block synthesis of virus completely in prolonged cultures could be explained as follows: The mean levels of DNA in inhibited cultures were within the normal range. This was shown in the present study by microspectrophotometry and previously(1) by the diphenylamine method. However, as shown with acridine orange, fluorescent antibody, and microspectrophotometric examination of material stained by Feulgen's method, synthesis of DNA, partial or complete, did occur in a few cells in each inhibited culture. The number of cells synthesizing DNA in FUDR-treated cultures was related to the amount of virus inoculated. When high titers of virus were added a larger number of these cells synthesizing DNA were present than when low titers of virus were inoculated. Apparently, a few cells in each culture were able to resist or adapt to FUDR, and produce infectious virus. The low yield of virus in cultures titrated at the earlier periods of incubation were probably due to the small number of cells that escaped the effects of FUDR at this time. The high yields of virus in cultures titrated after prolonged incubation were probably due to multiple cycles of virus multiplication, with each cycle causing an increase in the titer of the culture. After each cycle, with increased titer, more cells would be infected and more virus produced. Thus, in a prolonged culture the yield of virus would become nearly as high as that in a virus control culture. This explanation assumes that complete synthesis of DNA is not needed for production of virus. This is borne out by the present findings that only a small number of cells in inhibited cultures were producing DNA and virus, and in repeated observations with the fluorescent antibody method and acridine orange stain of the small amount of nuclear DNA actually associated with viral antigen.

Summary. 5-Fluorodeoxyuridine (FUDR) was only partially effective in suppressing synthesis of canine hepatitis virus in prolonged cultures of dog kidney cells. Suppression was more effective when cultures were inoculated with large amounts of virus than with small amounts of virus. Examinations of coverslip cultures by acridine orange, fluorescent antibody, and by microspectrophotometry of material stained by the Feulgen method revealed DNA and virus in a few cells in each FUDR-inhibited culture. Usually there was no increase in the mean level of DNA per inhibited culture. It was concluded that the multiplication of CH virus in the presence of FUDR occurred in a few resistant cells in each culture and after successive cycles of infection the titers became as high as in virus controls.

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