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RELATIONSHIP BETWEEN VIRUS MULTIPLICATION AND CULTURE CELLS SYNCHRONIZED BY EXCESS THYMIDINE TREATMENT IV. AUTORADIOGRAPHIC STUDIES OF CELLULAR AND VIRAL DNA SYNTHESES IN FL CELLS INFECTED WITH COWPOX VIRUS USING ^{3}H-DEOXYCYTIDINE

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SUMMARY Viral and cellular DNA syntheses were examined in FL cells treated with excess thymidine using autoradiography with ^{3}H-deoxycytidine in parallel with ^{3}H-thymidine. ^{3}H-deoxycytidine can be incorporated into nuclear and viral DNA of FL cells infected with poxvirus even in the presence of excess thymidine, while ^{3}H-thymidine cannot. The optimal concentration of excess thymidine for incorporation of ^{3}H-deoxycytidine into nuclear DNA was between 2.5×10^{-1} mM and 2.5×10^{-3} mM. Excess thymidine accumulated the cells in the early S phase, while excess deoxycytidine had no inhibitory effect on cell proliferation. Deoxycytidine at a concentration of between 0.01 mM and 0.02 mM completely prevented the block by 2.5 mM thymidine.

In cultures infected with poxvirus, cells showing both nuclear and viral DNA syntheses at the time of addition of excess thymidine remained in the S phase, but cells showing viral DNA synthesis before the S phase did not enter the S phase on addition of excess thymidine.

INTRODUCTION

In synchronized cultures of mammalian cells produced by addition of excess thymidine, which inhibits cellular DNA synthesis, thymidine-block can generally be released by washing out the thymidine (Xeros, 1962; Bootsma et al., 1964). The cells cannot be labeled with ^{3}H-thymidine in the presence of excess thymidine. Furthermore no cells are labeled with ^{3}H-thymidine for several hours after removal of excess thymidine, because of the large thymidine pool in the cells (Bootsma et al., 1964; Mantani et al., 1968; Mantani and Kato, 1970). Deoxycytidine releases the thymidine block and excess thymidine inhibits the formation of deoxycytidine (Xeros, 1962; Reichard et al., 1960; Morris and Fisher, 1960). Thus ^{3}H-

deoxycytidine, as a radioactive precursor of DNA, was expected to be incorporated into cellular DNA in the presence of, or soon after removal of excess thymidine.

Xeros (1962) using a Chang-appendix cell system, observed a burst of mitosis in cultures blocked for 24 hr with 2.0 mM excess thymidine after simply adding 2.0 mM excess deoxycytidine, which did not act as a brake on cell proliferation.

We studied the relationship between the host cell cycle and virus growth, with a poxvirus-FL cell system, in which the FL cells were synchronized by double treatment with excess thymidine. Our previous results (Mantani et al., 1968; Mantani and Kato, 1970; 1971;. 1972) were as follows:

1) When viral DNA synthesis began after the onset of the S phase, cellular DNA synthesis was partially, but not completely inhibited and proceeded during the period corresponding to the physiological S phase.

2) When viral DNA synthesis began during the Gl phase, cells did not enter the S phase.

3) When viral DNA synthesis began before the M phase, scarcely any cells entered mitosis.

4) When mitotic cells obtained in the presence of colchicine were infected with virus, neither viral DNA synthesis nor viral antigens, including virus induced cell surface antigen, were observed in them.

The above results were obtained using ^{3}H-thymidine. In the present work on the relationship between cellular and viral DNA syntheses soon after removal of thymidine-block and also in the presence of excess thymidine, we used ^{3}H-deoxycytidine since it may be incorporated into DNA in the presence of excess thymidine.

MATERIALS AND METHODS

1. *Vrius*

Cowpox virus (CPV) (LB red strain carrying the "A"v^{-} marker) was used throughout the experiments. The procedures used for preparation of virus materials and for plaque assays of CPV were described in the previous report (Mantani et al., 1970).

2. *Cells*

Eagle's minimum essential medium (MEM) supplemented with 10% calf serum was used as growth medium(GM). Cloned FL cells in Leighton tubes with cover glasses on the bottom were used throughout for autoradiography and FL cells in 50 ml prescription bottles were used to determine CPV growth curves. Cloned FL cells were prepared as described previously (Mantani et al., 1968). Cells were used one day after dispersion. All media contained penicillin (100 IU/ml), streptomycin(0.1 mg/ml) and terramycin(5 μg/ml). Cells were cultured at 37 C throughout the experiments.

3. *Chemicals*

Thymidine-6-^{3}H(^{3}H-Tdr) (specific activity 5 Ci/mM) and deoxycytidine-5-^{3}H(^{3}H-dCdr) (specific activity 10.4 Ci/mM) were purchased from Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan. Generally both these radioisotopes were used at a final concentration of 2 μCi/ml. Thymidine was from Nutritional Biochemical Co., deoxycytidine hydrochloride from Sigma Co., and colchicine krist. from Merck Co. Deoxyribonuclease 1(DNase) and ribonuclease (RNase) were purchased from Worthington Biochemical Corp., Freehold, New Jersey. Samples which had been labeled with ^{3}H-dCdr for 1 hr at 37 C and fixed with methanol were exposed to DNase(50 μg/ml in phosphate buffered saline containing 0.2 M $MgSO_4 \cdot 7H_2O$, pH 7.2) for 30 min at 37 C, or RNase(1 mg/ml) for 40 min at 40 C. Then, they were subjected to autoradiography.

4. *Virus growth and titration*

FL monolayer cells in 50 ml prescription bottles were inoculated with 0.2 ml of CPV material. After a 1 hr adsorption period, FL cells were washed with Hanks' solution and incubated with 5 ml of GM. The infected FL cells were taken out at appropriate times. CPV was assayed on BSCl cultures with agar overlay as described previously (Mantani et al., 1970).

5. *Autoradiography*

The procedure used for autoradiography was as described previously (Mantani et al., 1968, 1970) using exposure times of 3 or 6 days. Giemsa was employed for post-staining. The percentage of

labeled cells was estimated on at least 500 cells. Grain counts were made on 30 or 50 randomly selected nuclei. A Neopak microscope, model N(Olympus Co.), was used for microscopic obser-vaions, as reported by Rogers (1967).

6. *Scheme of experiments*

All experiments were schematically shown in Fig. 1.

RESULTS

1. *Comparison of the incorporations of ^{3}H-deoxycytidine and ^{3}H-thymidine into the nuclei of noninfected cells (Fig. 1, Experiment 1).*

As shown in Fig. 1, Expt. 1, a–f, FL cells in the Leighton tubes were incubated with 2 ml of various ^{3}H-labeled precursors of DNA (i.e. ^{3}H-Tdr (2 μCi/ml), ^{3}H-dCdr(2 μCi/ml), both ^{3}H-Tdr (2 μCi/ml) and ^{3}H-dCdr (2 μCi/ml), ^{3}H-Tdr (2 μCi/ml) in the presence of E-dCdr (0.1 mM), and ^{3}H-dCdr (2 μCi/ml) in the presence of E-Tdr (0.25 mM). The excess amounts of nucleosides were added at the same time as the ^{3}H-nucleosides. These samples were subjected to autoradiography using an exposure time of 6 days and the percentages of labeled nuclei were then calculated.

As shown in Table 1, between 24.0 and 26.4 percent of the nuclei in these samples were labeled. The average number of silver grains per nucleus, measured on 30 randomly selected labeled nuclei, was also calculated and is shown in Table 1. The average number of silver grains on nuclei labeled with ^{3}H-dCdr in the presence of 0.25 mM E-Tdr (189) was more than that on nuclei labeled with ^{3}H-dCdr only (73). On the contrary, the average number of silver grains on nuclei labeled with ^{3}H-Tdr in the presence of 0.1 mM E-dCdr (111) was less than that on nuclei labeled only with ^{3}H-Tdr (>200).

Treatment with RNase had no effect on the number of silver grains on nuclei labeled with ^{3}H-dCdr, while no silver grains were found after treatment of DNase. Thus the percentage of nuclei labeled with ^{3}H-dCdr reflects the percentage of cells showing nuclear DNA synthesis, but the incorporation of dCdr seems to

TABLE 1. *Percentages of labeled nuclei in randomly growing FL cells and average numbers of silver grains per nucleus in labeled nuclei. (Fig. 1, Experiment 1, a, b, c, d)*

Expt. 1.	Radio-isotope	Labeled nuclei %	Average number of silver grains per nucleus in labeled nuclei
a	^{3}H-dCdr	25.6	73
b	^{3}H-dCdr+E-Tdr	26.4	189
a	^{3}H-Tdr	24.6	>200
d	^{3}H-Tdr+E-dCdr	26.0	111
c	^{3}H-dCdr+^{3}H-Tdr	24.0	>200

E-Tdr: 0.25 mM, E-dCdr: 0.1 mM, ^{3}H-Tdr: 2μCi/ml, ^{3}H-dCdr: 2μCi/ml

TABLE 2. *Percentages of nuclei of FL cells labeled with ^{3}H-dCdr or ^{3}H-Tdr. The percentages were calculated using the same samples as for Figs. 2 and 3. (Fig. 1, Expt. 1, a, c, d, e, f)*

Expt. 1	Concentration of Tdr during labeling with ^{3}H-dCdr	Percentage of labeled nuclei
c	2.5 mM	20.6
c	2.5×10^{-1} mM	21.5
c	2.5×10^{-2} mM	23.5
c	2.5×10^{-3} mM	23.0
c	2.5×10^{-4} mM	21.5
c	2.5×10^{-5} mM	22.5
a	0	22.0
e	washed once with 2.5 mM E-Tdr	23.0
f	treated with 2.5 mM E-Tdr for 24 hr	92.6

Expt. 1	Concerntration of dCdr during labeling with ^{3}H-Tdr	Percentage of labeled nuclei
d	0.1 mM	23.1
d	0.01 mM	23.3
a	0	22.2

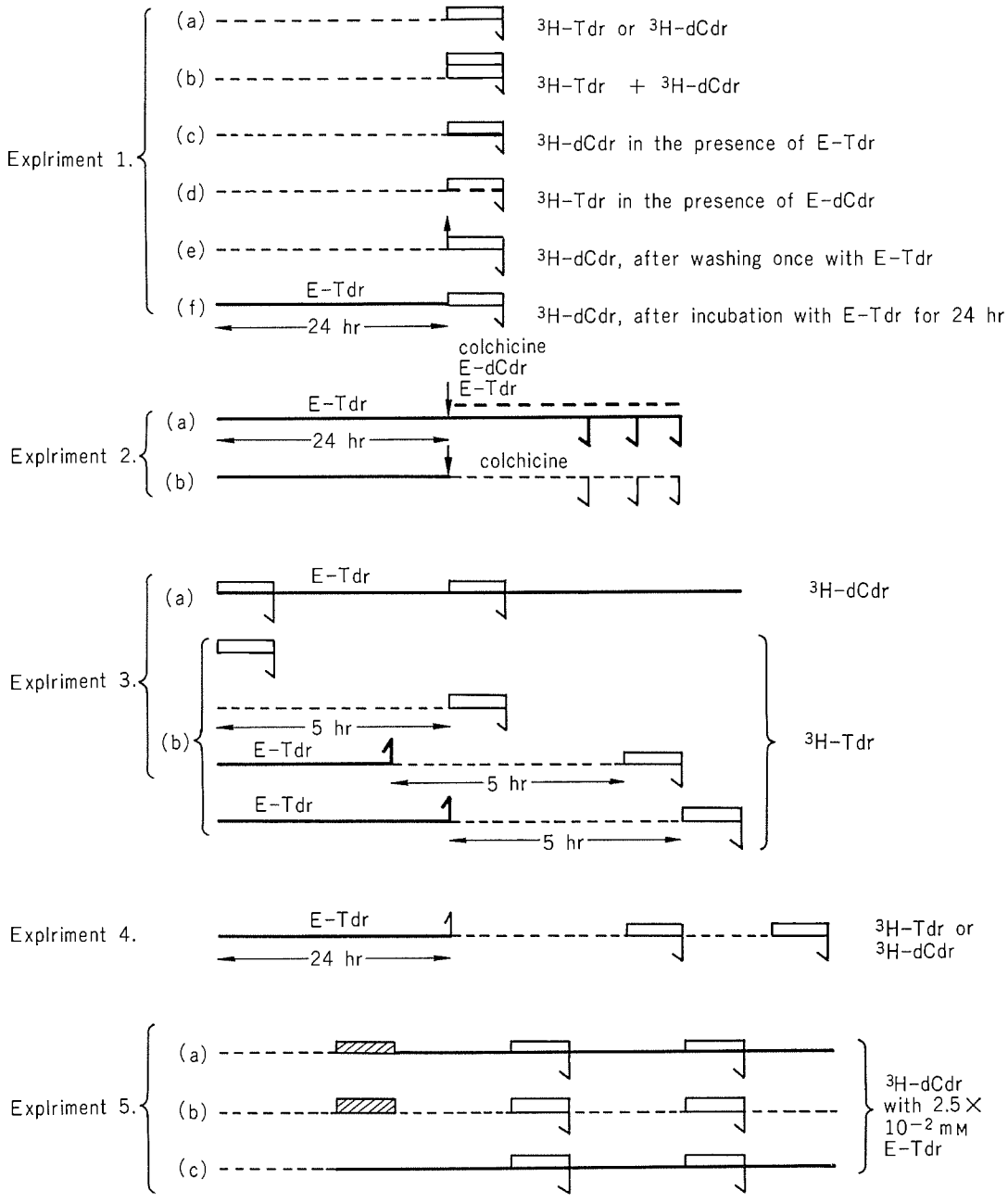
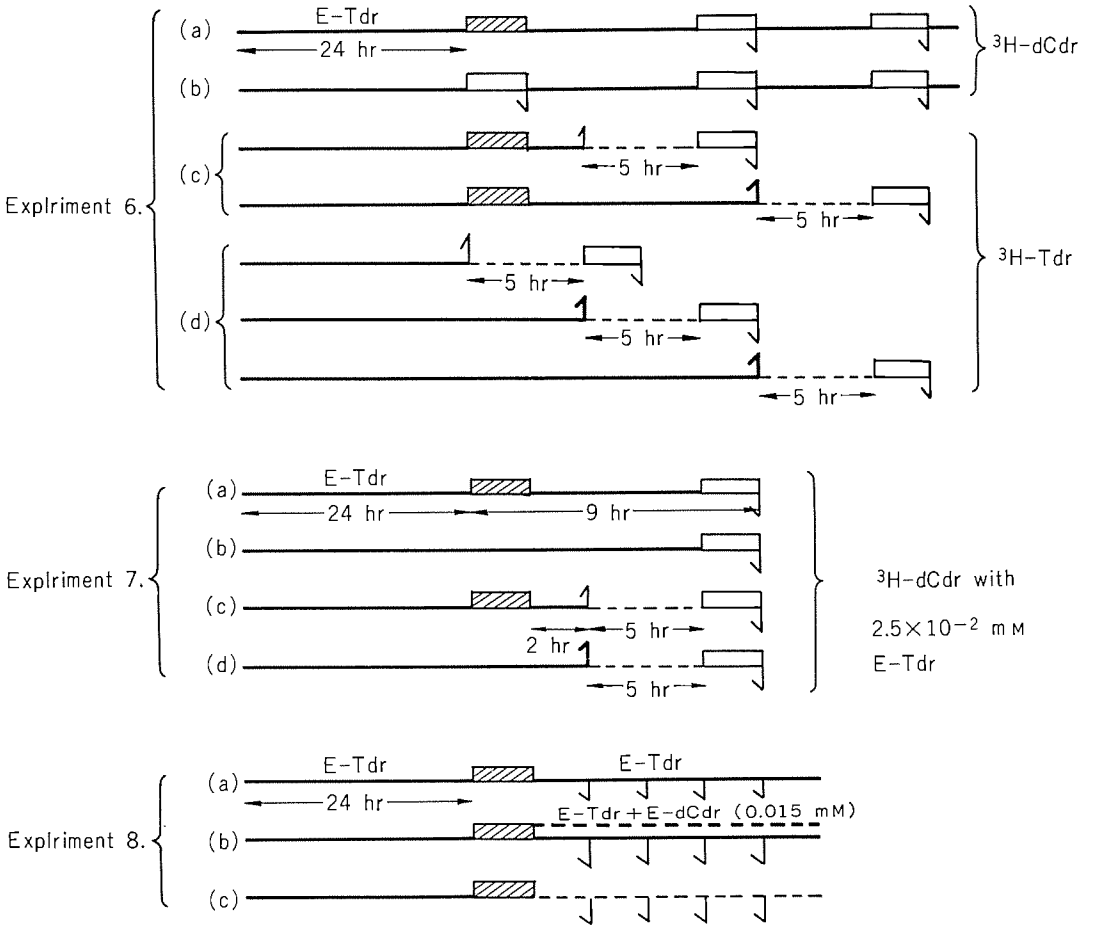


FIGURE 1. *Scheme of experiments*


Explriment 6.
(a)
E-Tdr
24 hr
(b)
(c)
5 hr
5 hr
(d)
5 hr
5 hr
5 hr
3H-dCdr
3H-Tdr
Explriment 7.
(a)
E-Tdr
24 hr
9 hr
(b)
(c)
5 hr
2 hr
(d)
5 hr
3H-dCdr with
2.5×10−2 m M
E-Tdr
Explriment 8.
(a)
E-Tdr
24 hr
E-Tdr
E-Tdr+E-dCdr (0.015 mM)
(b)
(c)
: Incubation with 3H-Tdr or 3H-dCdr for 1 hr
: One hr virus adsorption period
: Growth medium
: E-Tdr (2.5mM)
: E-dCdr
: Samples were taken out
: Washing with Hanks' solution
: Washing with E-Tdr
: Administration

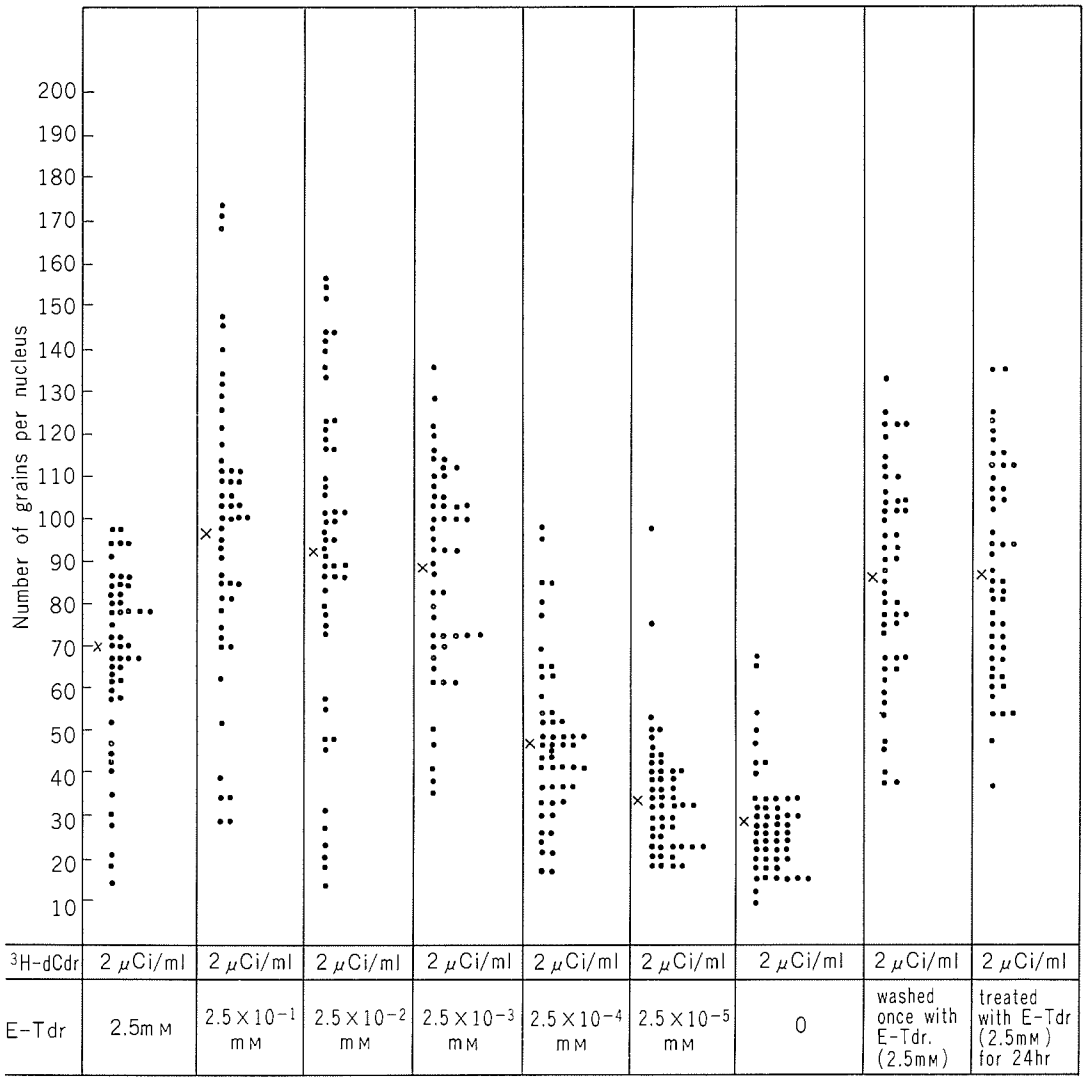


FIGURE 2. *Effects of various concentrations of E-Tdr upon the incorporation of ³H-dCdr (2 μCi/ml) into cellular DNA. Grain counts were made on 50 randomly selected labeled nuclei in the same samples as for Table 2.*

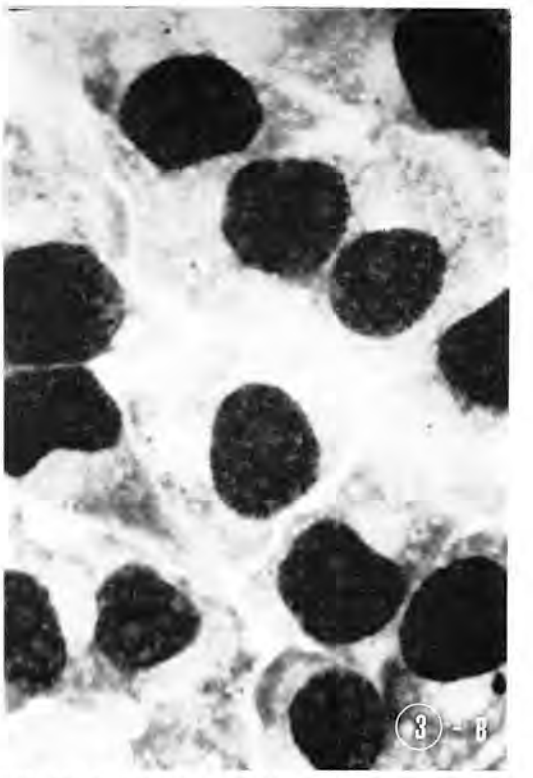


FIGURE 3 A and B. *Autoradiogram of FL cells labeled with ^{3}H-deoxycytidine (2 μCi/ml) in the presence of 2.5×10^{-2} mM excess thymidine. The cells seen in Fig. 3 A are the same cells as in Fig. 3 B. The number of silver grains per nucleus in Fig. 3 A is greater than that observed in Fig. 4 A.*

be influenced by the presence of Tdr and *vice versa*.

Based on the results shown in Table 1, the effect of various concentrations of Tdr on the incorporation of ^{3}H-dCdr (2 μCi/ml) into the nucleus was investigated as shown in Fig. 1, Expt. 1, c. The effect of E-dCdr concentration on nuclear incorporation of ^{3}H-Tdr (2 μCi/ml) was also investigated at the same time (Expt. 1, d). FL cells were incubated either with ^{3}H-dCdr (2 μCi/ml) for 1 hr in the presence of various concentrations of E-Tdr, or with ^{3}H-Tdr (2 μCi/ml) in the presence of various concentrations of E-dCdr. In Expt. 1, e, FL cells grown in the growth medium (GM) were washed once with 2.5 mM E-Tdr and then incubated with ^{3}H-dCdr (2 μCi/ml) for 1 hr. In Expt. 1, f, FL cells were incubated with 2.5 mM E-Tdr for 24 hr and then directly with ^{3}H-dCdr (2 μCi/ml) for 1 hr without washing with Hanks' solution. All samples were autoradiographed using an exposure period of 3 days.

The results of these experiments are shown in Table 2. Between 20.6 and 23.5 percentage of the nuclei, were labeled with either ^{3}H-dCdr or ^{3}H-Tdr irrespective of the concentration of of either Tdr or dCdr. In Expt. 1, f, 92.6 percent of the nuclei were labeled because, due to thymidine-block, cells accumulated in the S phase. The number of grains on 50 randomly selected labeled nuclei in these samples was calculated and the number per nucleus is plotted as shown in Fig. 2. When cells were labeled with ^{3}H-dCdr in the presence of E-Tdr

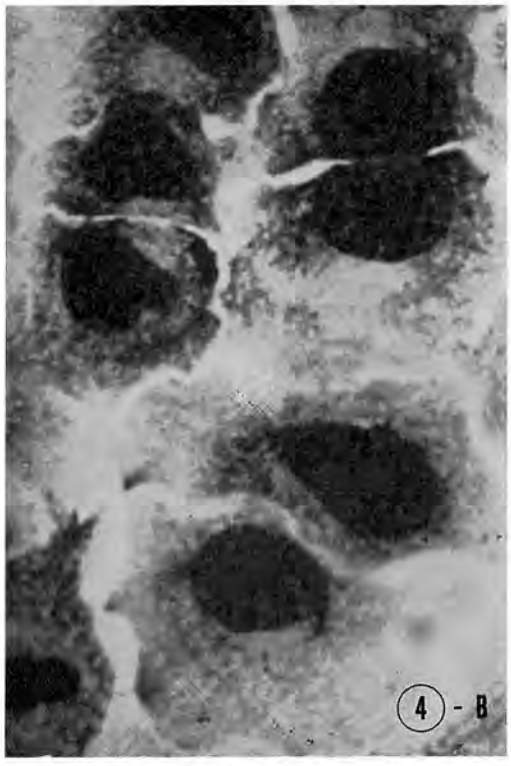


FIGURE 4 A and B. *Autoradiogram of FL cells labeled with ^{3}H-deoxycytidine (2 μCi/ml) for 1 hr at 37 C. The photos in A and B were taken with a Neopak microscope, model N(Olympus Co., Japan). The cells in Fig. 4 A, in which the light source is from above, are the same cells as in those Fig. 4 B, in which the light goes through the specimen from below. Silver grains appear bright (in a dark field) in Fig. 4 A.*

at concentrations of between 2.5×10^{-1} mM and 2.5×10^{-3} mM, the average number of silver grains per nucleus was between 90 and 100. Fig. 3 showed an autoradiogram of the cells. When cells were labeled with ^{3}H-dCdr after being washed once with 2.5 mM E-Tdr or after treatment with 2.5 mM E-Tdr for 24 hr, the average number of grains per nucleus was also about 90.

The average number of silver grains per nucleus was lowest in cells which were labeled with ^{3}H-dCdr only in the absence of E-Tdr, being 28, as shown in Fig. 4. When cells were incubated with ^{3}H-dCdr in the presence of 2.5 mM E-Tdr, the average number of grains per nucleus was 70.

These results indicate that the incorporation of ^{3}H-dCdr into nuclear DNA was accelerated by the presence of E-Tdr and the optimal concentration of E-Tdr for the incorporation of ^{3}H-dCdr into nuclear DNA was between 2.5×10^{-1} mM and 2.5×10^{-3} mM. Moreover the concentration of E-Tdr in the medium when cells are washed once with 2.5 mM E-Tdr and then incubated in 2 ml of medium also seems to be between 2.5×10^{-1} mM and 2.5×10^{-3} mM, judging from the grain number per nucleus. As shown in Fig. 5, when cells were labeled with ^{3}H-Tdr (2 μCi/ml), the average number of grains per nucleus in the presence of E-dCdr (0.1 mM and 0.01 mM) was less than that in the absence of E-dCdr.

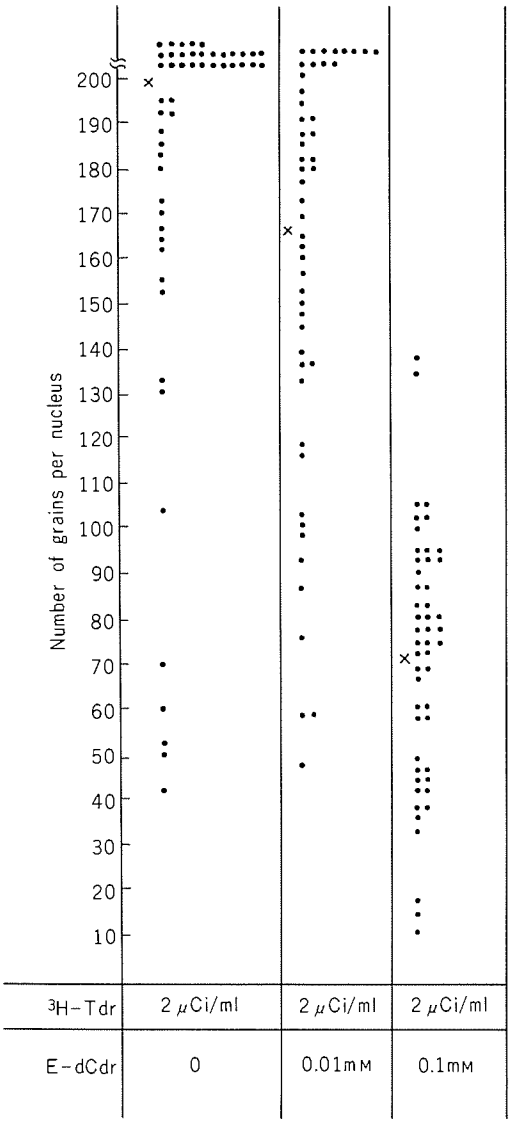


FIGURE 5. *Effects of various concentrations of E-dCdr upon the incorporation of ^{3}H-Tdr (2 μCi/ml) into cellular DNA. The incorporation of ^{3}H-Tdr was inhibited by the addition of E-dCdr.*

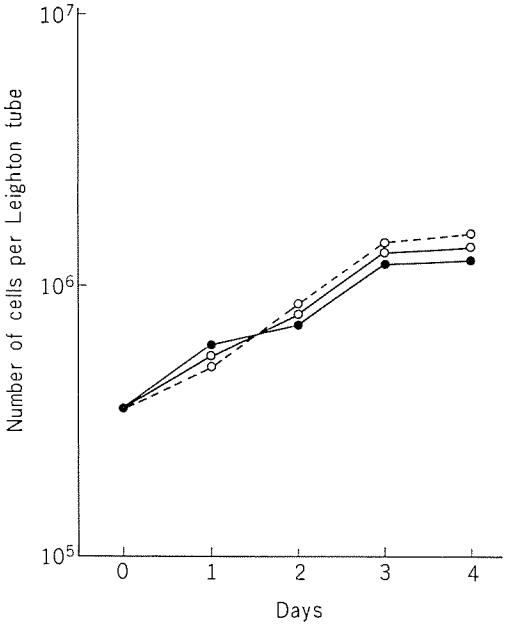


FIGURE 6. *Effects of E-dCdr on cell proliferation. FL cells were dispersed to the Leighton tubes. These cells were incubated with GM or GM containing 0.1 mM E-dCdr, or GM containing 1 mM E-dCdr. The number of cells per Leighton tube was calculated daily and plotted.* ○·····○, *GM;* ○———○, *GM containing 0.1 mM E-dCdr;* ●———●, *GM containing* 1 *mM E-dCdr.*

E-Tdr inhibits FL cell proliferation, as described in a previous report (Mantani et al., 1970). However, 1 mM or 0.1 mM E-dCdr, which from the results shown in Fig. 5, were expected to inhibit DNA synthesis had no effect on cell proliferation (Fig. 6). These results indicate that nuclear DNA synthesis may not be inhibited by the presence of a high concentration of E-dCdr (up to 1 mM), because cell growth is not inhibited by E-dCdr, but that incorporation of ^{3}H-Tdr into nuclear DNA is inhibited by the presence of a high concentration of E-dCdr.

2. *Synchronized cell growth in the presence of excess thymidine studied using deoxycytidine (Fig. 1, Expt. 2).*

As shown in Fig. 1, Expt. 2, a and Fig. 7, FL cells were incubated with GM containing 2.5 mM E-Tdr for 24 hr and then with fresh GM containing 2.5 mM E-Tdr, 5×10^{-8} M colchicine and various concentrations of E-dCdr (0~0.5 mM). As a control, FL cells treated with 2.5 mM E-Tdr for 24 hr were washed three times with Hanks' solution to obtain a synchronized culture (Mantani et al., 1968; Mantani and Kato, 1970, 1971) and then incubated with fresh GM containing colchicine (Expt. 2, b). FL cells were taken out 13, 16 and 19 hr after addition of colchicine. The samples were dried, fixed and stained with Giemsa and their mitotic indices were calculated. As shown in Fig. 7, the number of mitotic cells in the presence of both 2.5 mM E-Tdr and either 0.02 or 0.01 mM E-dCdr increased at the same rate as those in the absence of E-Tdr. However, the number of mitotic cells in the presence of both 2.5 mM E-Tdr and other concentrations of E-dCdr did not increase. These results indicate that the inhibition of cellular DNA synthesis by 2.5 mM E-Tdr is prevented by addition of E-dCdr at concentrations between 0.01 and 0.02 mM. The results also show that the inhibitory effect of E-Tdr on cell proliferation can be reversed by addition of E-dCdr.

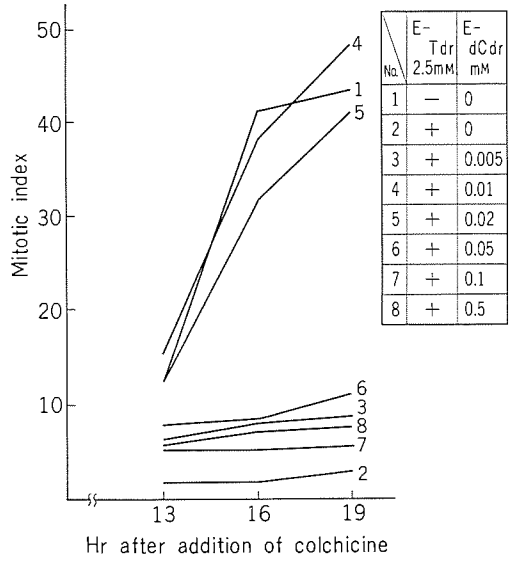


FIGURE 7. *Effects of various concentrations of E-dCdr upon FL cell-proliferation in the presence of E-Tdr (2.5 mM) accumulated in the early S phase by treatment with E-Tdr (2.5 mM) for 24 hr. The procedure was that of Experiment 2 of Fig. 1.*

3. *Cellular DNA synthesis of cells accumulated in the S phase by addition of 2.5 mM E-Tdr (Fig. 1, Experiment 3).*

As shown in Fig. 1, Expt. 3, FL cells were pulse labeled with either ^{3}H-dCdr or ^{3}H-Tdr at appropriate times after addition of E-Tdr

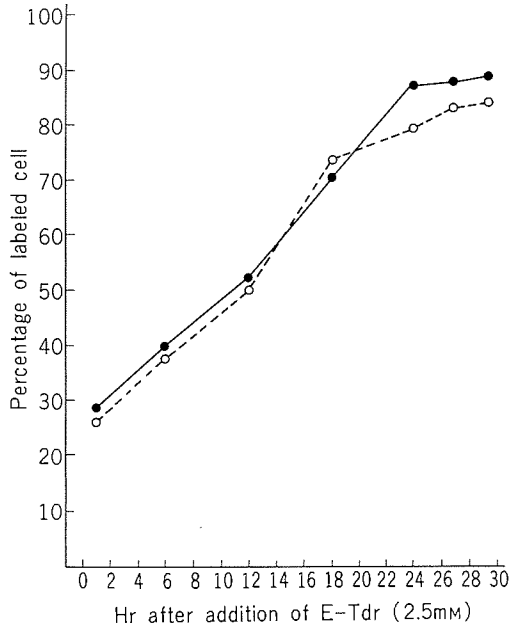


FIGURE 8. *Percentage of labeled nuclei incorporating either ^{3}H-dCdr (●——●) or ^{3}H-Tdr (○·····○) during a 1 hr labeling period. The procedures were those of Experiment 3 of Fig. 1. The number of labeled nuclei increased with time after addition of E-Tdr (2.5 mM).*

(2.5 mM) (Expt. 3, a, b). To label these cells with ^{3}H-Tdr, the cells have to be cultured in medium free from E-Tdr for at least 5 hr before labelling, as described in a previous report (Mantani and Kato, 1970). Fig. 8 shows that the percentage of cells labeled with ^{3}H-dCdr increased in parallel with those labeled with ^{3}H-Tdr until 30 hr after addition of E-Tdr. Twenty four hr after addition of E-Tdr, more than 85% of the FL cells were in the S phase.

In this experiment 27 percent of the FL cells which were in the S phase at the beginning of incubation with E-Tdr did not proceed further in this phase because of the E-Tdr-block while other cells continued through their life cycle until they are accumulated in the early S phase, as described in the previous report (Studzinski et al., 1968). Thus it would appear that the period necessary for maximum accumulation in the S phase agrees with the doubling period of FL cells.

4. *Cellular DNA synthesis of cells after removal of E-Tdr (Fig. 1, Experiment 4).*

As shown in Fig. 1, Expt. 4 and Fig. 9, FL cells incubated with 2.5 mM E-Tdr for 24 hr were washed three times with Hanks' solution and pulse labeled with either ^{3}H-dCdr or ^{3}H-Tdr at appropriate times after removal of E-Tdr. Fig. 9 shows that FL cells were well synchronized. The mitotic index was 17.5 percent 15 hr after removal of E-Tdr.

When cells were pulse labeled with ^{3}H-dCdr soon after removal of E-Tdr, more than 90 percent of the cells were in the S phase. When cells were pulse labeled with ^{3}H-Tdr, the percentage of labeled cells increased rapidly from 0 to more than 90 percent, 6 hr after removal of E-Tdr. The difference between the percentages of cells labeled with ^{3}H-dCdr and with ^{3}H-Tdr within 6 hr after removal of E-Tdr may depend on the thymidine pool in the cells after removal of E-Tdr. It would appear that cells labeled with ^{3}H-dCdr just after removal of E-Tdr were in the early S phase and that those labeled with ^{3}H-Tdr 6 hr after removal of E-Tdr were in the middle of the S phase.

Based on the above results, we used 2 μCi/ml of ^{3}H-dCdr, as a labeled precursor in the presence of E-Tdr (2.5×10^{-1} mM$\sim 2.5 \times 10^{-3}$ mM) to investigate the relationship between cellular and viral DNA syntheses in cells infected with cowpox virus.

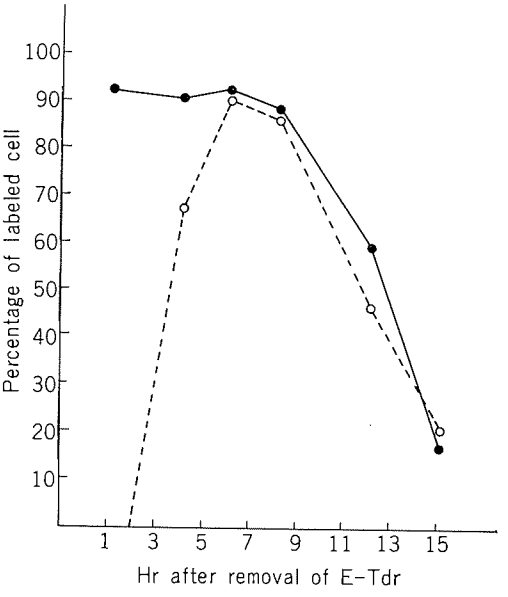


FIGURE 9. *Percentage of labeled nuclei incorporating ^{3}H-dCdr (●——●) or ^{3}H-Tdr (○·····○) during a 1 hr labeling period. FL cells were incubated with 2.5 mM E-Tdr for 24 hr, before the experiment as shown in Experiment 4 of Fig. 1. FL cells were not labeled with ^{3}H-Tdr until 4 hr after removal of E-Tdr by washing with Hanks' solution, but they were well labeled with labeled with ^{3}H-dCdr soon after removal of E-Tdr.*

5. *Nuclear DNA synthesis in cells infected with cowpox virus in the presence of 2.5 mM E-Tdr (Fig. 1, Experiment 5).*

Two experiments (Fig. 1, Expt. 5,) were designed to study this and results are shown in Figs. 10 and 11. FL cells were infected with CPV. The multiplicity of infection (moi) was 100 in the experiment shown in Fig. 10, and 3 in the experiment shown in Fig. 11. After a one hr adsorption period, FL cells were incubated in either GM containing 2.5 mM E-

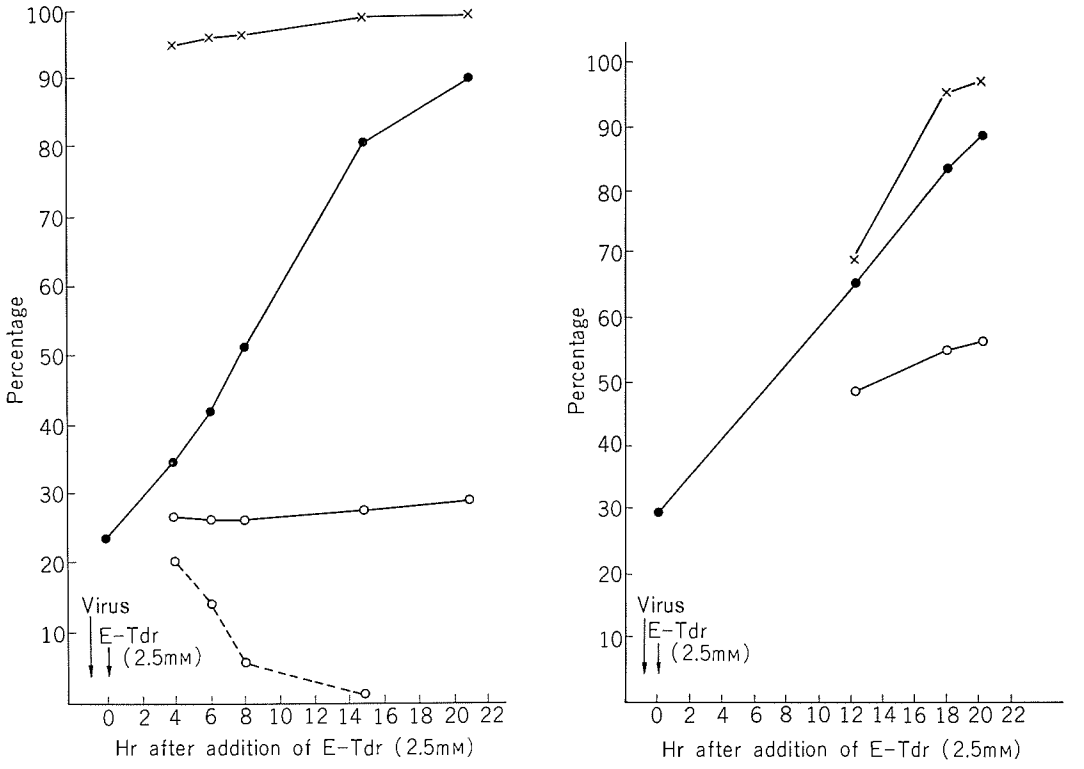


FIGURE 10 and 11. *Effect of virus infection upon ac- cumulation of FL cells in the S phase by E-Tdr (2.5 mM)-block. (100 moi in Fig. 8 and 3 moi in Fig. 9).* ×———×, *percentage of "B" cells;* ●———●, *percentage of labeled nuclei in noninfected control cells incubated with E-Tdr;* ○———○, *percentage of labeled nuclei in "B" cells incubated with E-Tdr after infection;* ○·····○, *percentage of labeled nuclei in "B" cells, incubated with GM after infection. Noninfected cells accumulated in the S phase due to 2.5 mM E-Tdr block and the percentage of labeled cells was 90, 22 hr after addition of E-Tdr. In the case of infected cells, the percentage of labeled nuclei in "B" cells remained between 20 and 30 in Fig. 8 and between 50 and 55 in Fig. 11, after the percentage of "B" cells reached 90. The percentage of labeled nuclei in "B" cells in the absence of E-Tdr decreased gradually because of inhibition of the cell cycle by virus infection.*

Tdr (Expt. 5, a) or GM only (Expt. 5, b). Noninfected FL cells were also incubated in GM containing 2.5 mM E-Tdr (Expt. 5, c). At appropriate times, GM or GM containing E-Tdr was replaced by 2 ml of MEM containing both ^{3}H-dCdr (2 μCi/ml) and 2.5×10^{-2} mM E-Tdr and incubation was continued for 1 hr. Then samples were taken out for autoradiography. All " B " type inclusions were well labeled. The percentage of labeled nuclei in the noninfected samples and the percentage of cells bearing " B " type inclusions (" B " cells) in the infected samples were calculated. As shown in Figs. 8, 10 and 11, the number of noninfected, control cells in the S phase increased rapidly until 22–24 hr after addition of E-Tdr. In the first experiment using a moi of 100 (Fig. 10) " B " type inclusions were observed in more than 90% of the cells 5 hr after infection. The percentage of " B " cells with labeled nuclei in the presence of E-Tdr remained almost constant (25% to 27%) from

4 to 22 hr after addition of E-Tdr, while the percentage of " B " cells with labeled nuclei in the absence of E-Tdr decreased. In a second experiment a lower input of virus was used (3 moi) so that the percentage of " B " cells did not reach 90 until 19 hr after infection (Fig. 11). In this experiment the percentage of " B " cells with labeled nuclei remained constant (55%–57%) from 19 hr to 21 hr after infection. In Figs. 10 and 11, if " B " cells in the Gl phase could enter the S phase, the curve of the percentage of " B " cells with labeled nuclei in the presence of E-Tdr, would be parallel with that of noninfected cells with labeled nuclei in the persentage of E-Tdr. The percentage of cells showing both nuclear and viral DNA synthesis among the " B " cells in the presence of E-Tdr neither decreased nor increased when almost all the cells showed viral DNA synthesis in the cytoplasm. Thus in the cells showing both, nuclear and viral DNA synthesis nuclear DNA synthesis probably began before the onset of viral DNA synthesis, and these cells probably remained in the S phase due to inhibition of nuclear DNA synthesis by E-Tdr. The other cells which showed viral DNA synthesis in the cytoplasm before the S phase probably could not enter the S phase. This agrees with the conclusion reached in a previous report (Mantani et al., 1970) from autoradiographic studies with ^{3}H-Tdr. As shown in Fig. 10, when infected cells were incubated in the absence of E-Tdr, the number of cells showing both nuclear DNA and viral DNA synthesis decreased rapidly, as described in a previous report (Mantani et al., 1968).

6. *Nuclear DNA synthesis of cells infected with cowpox virus after treatment with E-Tdr* for 24 *hr* (*Fig.* 1, *Experiments* 6, 7).

As shown in Expt. 6 of Fig. 1, FL cells were infected with CPV after incubation with GM containing 2.5 mM E-Tdr for 24 hr. After virus adsorption, cells were reincubated with E-Tdr. These infected cells were then pulse labeled either with ^{3}H-dCdr (2 μCi/ml) (Expt. 6, a) or ^{3}H-Tdr (2 μCi/ml) (Expt. 6, c) for 1 hr, 17, 20 and 28 hr after infection. Noninfected cells were also labeled similarly (Expt, 6, b, d). To labele these cells with ^{3}H-Tdr, the cells have to be cultured in medium free from E-Tdr for at least 5 hr before labelling, as described in a previous report (Mantani and Kato, 1970). These samples were subjected to autoradiography using an exposure period of 6 days. The percentages of labeled nuclei in noninfected, control cells and labeled nuclei of " B " cells are shown in Fig. 12. The num-

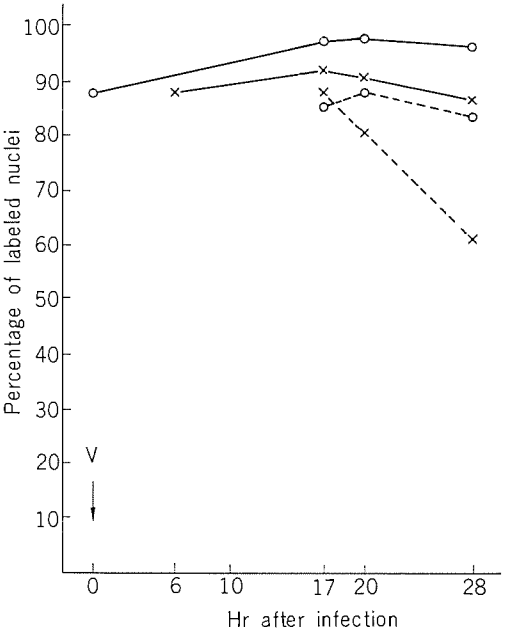


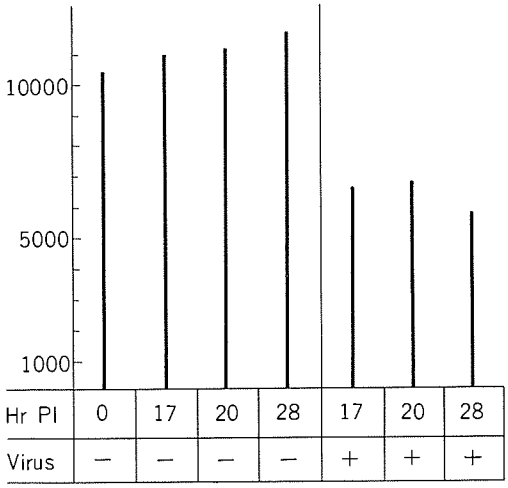
FIGURE 12. *Effect of viral DNA synthesis upon nuclear DNA synthesis of FL cells accumulated in the S phase by E-Tdr treatment for 24 hr. (moi 50)* ○——○*: percentage of labeled nuclei in noninfected cells labeled with ^{3}H-dCdr soon after removal of E-Tdr;* ○·····○*, percentage of labeled nuclei in " B " cells labeled with ^{3}H-dCdr soon after removal of E-Tdr;* ×——×*, percentage of labeled nuclei in noninfected cells labeled with ^{3}H-Tdr 5 hr after removal of E-Tdr by washing;* ×·····×*, percentage of labeled nuclei in " B " cells labeled with ^{3}H-Tdr 5 hr after removal of E-Tdr. The percentage of " B " cells in the infected cells was more than 90, 17 hr after infection.*

ber of grains on 50 randomly selected nuclei were counted and the total number of grains per 50 nuclei are plotted in Figs. 13 and 14. The percentage of " B " cells was already more than 90, 17 hr after infection.

In the experiment with ^{3}H-dCdr many nuclei of " B " cells were well labeled as shown in Fig. 15. The curve of the percentage of " B " cells with labeled nuclei is close to that of noninfected cells with labeled nuclei (Fig. 12). The total numbers of silver grains per 50 randomly selected nuclei of " B " cells are less than those of noninfected cells (Fig. 13). These results may indicate the effect of viral DNA synthesis upon nuclear DNA synthesis of cells in the early S phase.

In the experiment with ^{3}H-Tdr, the curves of the percentages of " B " cells with labeled nuclei decreased from 87%, 17 hr after infection to 60%, 28 hr after infection, although the percentage of noninfected cells with labeled nuclei remained almost constant at more than 84% throughout. The inhibition of nuclear DNA synthesis in " B " cells was remarkable, when the total number of silver grains per 50 nuclei in " B " cells was compared with that in noninfected, control cells (Fig. 14). These results may indicate an effect of viral DNA synthesis upon nuclear DNA synthesis of cells in the middle of the S phase, since these cells were labeled with ^{3}H-Tdr 6 hr after removal of E-Tdr. Therefore, the effect of viral DNA synthesis upon nuclear DNA synthesis of cells in the middle of the S phase is much larger than that in the early S phase.

To confirm this results, another set of experiments was designed in which infected and noninfected cells were pulse labeled with ^{3}H-dCdr both in the early S phase and in the middle of the S phase (Fig. 1, Expt. 7). FL cells were inoculated with CPV (20 moi), after 24 hr incubation with E-Tdr. One hr was allowed for virus adsorption. In the experiment on pulse labeling in the early S phase, infected cells were reincubated with E-Tdr and taken out 9 hr after infection after being pulse labeled with ^{3}H-dCdr (2 μCi/ml) in the presence of 2.5×10^{-2} mM E-Tdr for 1 hr, soon after removal of E-Tdr (Fig. 1, Expt. 7, a). The percentages of " B " cells was 80%. Simultane-



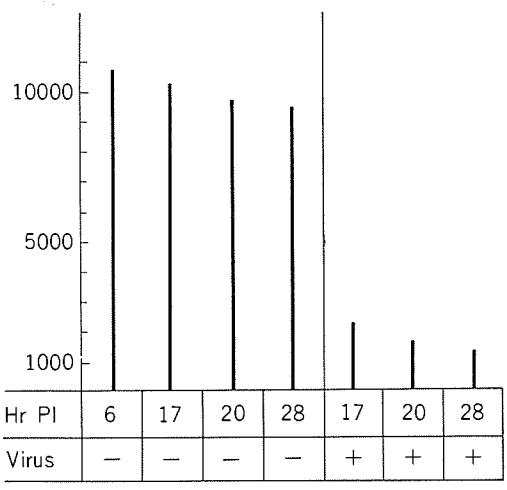
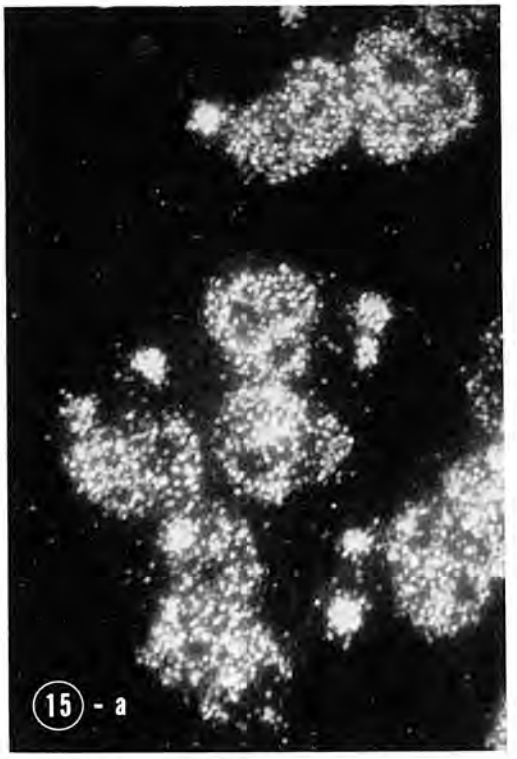


FIGURE 13 and 14. *Effect of viral DNA synthesis upon nuclear DNA synthesis of FL cells accumulated in the S phase by E-Tdr treatment for 24 hr.*
Fig. 13, Total number of silver grains per 50 nuclei labled with ^{3}H-dCdr (cells in early S phase).
Fig. 14, Total number fo silver grains per 50 nuclei labeled with ^{3}H-Tdr (cells in the middle of the S phase).

ously, noninfected cells cultured in E-Tdr were also pulse labeled with ^{3}H-dCdr in the presence of 2.5×10^{-2} mM E-Tdr for 1 hr (Fig. 1, Expt. 7, b). In the experiment on pulse labeling in the middle of the S phase, infected cells were washed with Hanks' solution after reincubation with E-Tdr for 2 hr and then incubated with GM for 5 hr. Then, these cells were pulse labeled for 1 hr with ^{3}H-dCdr in the presence of 2.5×10^{-2} mM E-Tdr. The samples were taken out 9 hr after infection (Fig. 1, Expt. 7, c). The percentage of " B " cells was 88%. Simultaneously noninfected cells were also pulse labeled with ^{3}H-dCdr in the presence of 2.5×10^{-2} mM E-Tdr for 1 hr (Fig. 1, Expt. 7, d).

The numbers of silver grains per nucleus in these samples were calculated on 50 randomly selected nuclei and plotted (Fig. 16). In noninfected, control cells the average number of grains per nucleus in the middle of the S phase was more than in the early S phase. However, the average number of grains on the nuclei of " B " cells was less than that on the nuclei of noninfected cells and the inhibition of nuclear DNA synthesis in the middle of the S phase of " B " cells was more than that in the early S phase.

Fig. 12 also shows evidence at a cellular level that E-Tdr accumulates in cells in the early S phase, as concluded previously from the results of biochemical experiments (Schaer et al., 1971; Studzinski and Lambert, 1969). After 24 hr treatment with 2.5 mM E-Tdr, more than 80%



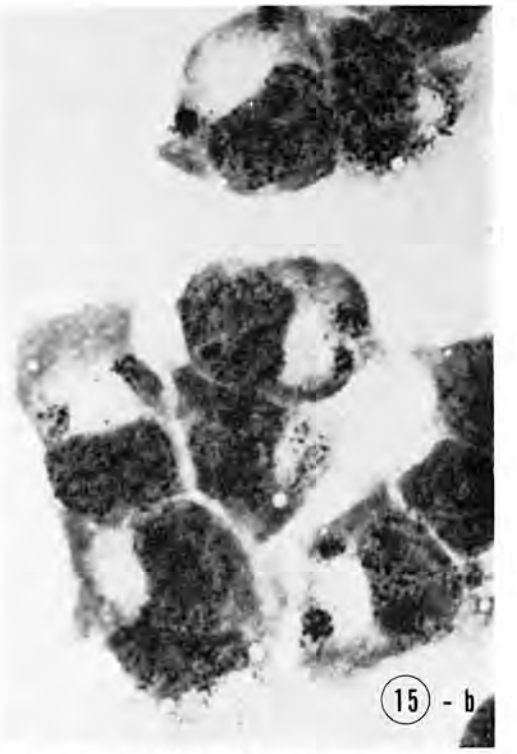


FIGURE 15 A and B. *Autoradiogram of FL cells infected with cowpox virus, labeled with 2 μCi/ml ^{3}H-deoxycytidine for 1 hr in the presence of 2.5×10^{-2} mM excess thymidine. The sample was taken out for autoradiography 17 hr after infection and 1 hr after removal of thymidine treatment for 41 hr. The cells in Fig. 17 A, are the same cells as those in Fig. 17 B. Both nuclei and " B " type inclusions have many silver grains on them.*

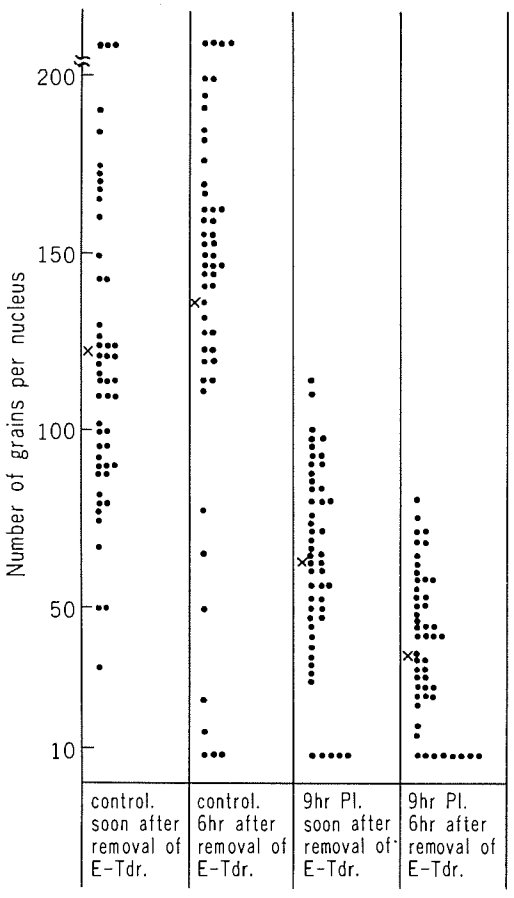


FIGURE 16. *Effect of viral DNA synthesis upon nuclear DNA synthesis of FL cells accumulated in the S phase by E-Tdr treatment for 24 hr. (moi 20). Both infected and noninfected FL cells were pulse labeled with ^{3}H-dCdr in the presence of 0.025 mM E-Tdr for 1 hr, 7 hr after infection either soon after removal of E-Tdr, in early S phase or 5 hr after removal of E-Tdr, in the middle of the S phase. Grain counts were made on 50 randomly selected nuclei of the sample. The inhibition of nuclear DNA synthesis of " B " cells was more sensitive in the middle of the S phase than that in the early S phase. The percentage of " B " cells was 78% in the early S phase and 88% in the middle of the S phase.*

of the noninfected cells may be in the S phase, since these cells were well labeled with ^{3}H-dCdr. However, the possibility that these labeled cells entered the S phase from the G1 phase during the 1 hr labeling period cannot be excluded. As described before, " B " cells could not enter the S phase, when viral DNA synthesis started before the S phase. In the experiment shown in Fig. 12, cells which had been treated with 2.5 mM E-Tdr for 24 hr were infected with CPV and reincubated with 2.5 mM E-Tdr. Even if the cells arrested in the G1 phase by an E-Tdr block entered the S phase during the 1 hr labeling period in the experiment with ^{3}H-dCdr, " B " cells should be unable to enter the S phase because of virus infection. However, 85% of the " B " cells were in the S phase 17 hr after infection, indicating that 2.5 mM E-Tdr must arrest the cells in the early S phase, instead of the G1 phase.

7. *Effect of excess deoxycytidine upon virus growth in the presence of* 2.5 *mM E-Tdr (Fig.* 1, *Expt.* 8).

FL monolayer cells in 3 series of 50 ml prescription bottles were inoculated with 0.2 ml of CPV (0.1 moi) after treatment with 2.5 mM E-Tdr for 24 hr as shown in Fig. 1, Expt. 8. After a 1 hr period of adsorption of virus, cells were washed with Hanks' solution. Then 5 ml of GM containing 2.5 mM E-Tdr was again added to one series of bottles (Expt. 8, a). Five ml of GM containing both 2.5 mM E-Tdr and 0.015 mM E-dCdr, which should release the E-Tdr-block of nuclear DNA synthesis, judging from the experiment in Fig. 6, were added to the second series of bottles (Expt. 8, b). Five ml of GM were added to the third series of bottles (Expt. 8, c). At appropriate times, the samples were taken out and the infectivity of virus (PFU) was titrated and plotted (Fig. 17).

CPV grew well in the presence of E-Tdr, but the virus yield was lower than that in the presence of both E-Tdr and E-dCdr, being almost identical with that in GM only.

The similarity in the virus yields in the latter two series can be explained by release of the 2.5 mM E-Tdr block of nuclear DNA synthesis by addition of 0.015 mM E-dCdr.

When various concentrations (0.0015 mM,

0.015 mM and 0.15 mM) of E-dCdr were added to FL monolayer cells infected with CPV, virus yields were similar to those in the absence of both E-dCdr and E-Tdr for up to 24 hr after infection. Thus E-dCdr did not inhibit either cell proliferation or virus growth.

DISCUSSION

Excess thymidine (E-Tdr) inhibits the conversion of cytidine to deoxycytidine (dCdr) (Reichard et al., 1950; Morris and Fisher, 1960). Xeros (1962) and Bootsma et al. (1964) found that double treatment of cells with E-Tdr was an efficient method for synchronizing cultures. This method was introduced into virology to study the relationship between virus growth and the host cells cycle (Groyon and Kniazeff, 1967; Mantani et al., 1968, 1970; Mantani and Kato, 1970, 1971; Hodge and Scharff, 1969; Ensminger and Tamm, 1970; Cohen et al., 1971; Lawrence, 1971). The relationship between nuclear DNA and poxvirus DNA synthesis has been studied at a cellular level by quantitative autoradiography of ^{3}H-thymidine (Kato et al., 1962, 1964; Mantani et al., 1968). However, it is difficult to label nuclear and viral DNA with ^{3}H-thymidine in the presence of E-Tdr. Furthermore, cells which had been treated with E-Tdr for 24 hr could not be labeled with ^{3}H-Tdr within at least 5 hr after removal of ^{3}H-Tdr, because the intracellular thymidine pool was large (Bootsma et al., 1964; Mantani et al., 1968, 1970; Mantani and Kato, 1970). In our experiments with E-Tdr, autoradiography with ^{3}H-dCdr made it possible to follow DNA synthesis in the presence of E-Tdr or soon after its removal.

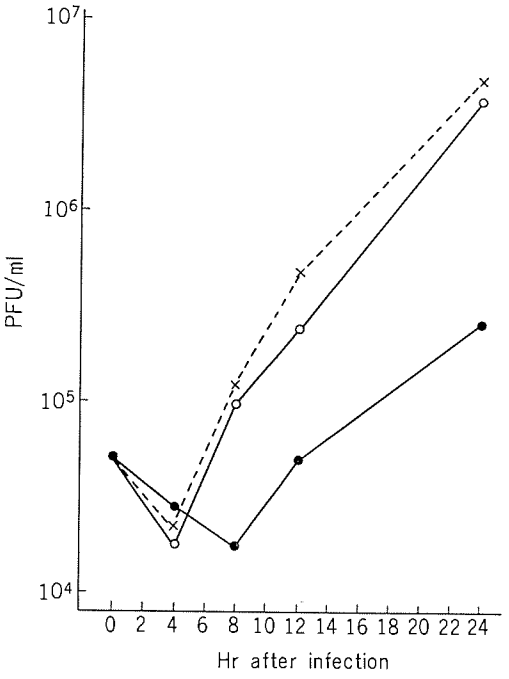


FIGURE 17. *Virus multiplication in FL cells in the presence of 2.5 mM E-Tdr and 0.015 mM E-dCdr.*

FL cells treated with 2.5 mM E-Tdr for 24 hr, were infected with cowpox virus. ●——●, *Infected cells kept in the presence of E-Tdr;* ○——○, *Infected cells kept in the presence of E-Tdr and 0.015 mM E-dCdr;* ×——×, *Infected cells kept in GM only.*

Almost all cells treated with E-Tdr for 24 hr were well labeled with ^{3}H-dCdr. There are two possibilities as to the phase of cells after treatment with E-Tdr for 24 hr. One possibility is that all the cells are in the S phase. The other possibility is that all cells are in the G1 phase and that ^{3}H-dCdr causes them to enter the S phase. Experiments with cowpox virus (CPV) were carried out to study these possibilities. As reported before, E-Tdr inhibits nuclear DNA synthesis but does not inhibit DNA synthesis of poxvirus appreciably (Mantani et al., 1970). Cells were treated with E-Tdr for 24 hr and then infected with CPV. After development of " B " inclusions in the presence of E-Tdr these cells were labeled with ^{3}H-dCdr. Autoradiography showed that the nuclei and " B " inclusions of nearly all cells were well labeled. Cells in the G1 phase, in which viral DNA synthesis had been initiated, could not enter the S phase (Mantani and Kato, 1970, 1971). These data, therefore, support the idea that cells treated with E-Tdr are in the S phase, rather than the G1 phase (Studzinski and Lambert, 1968; Bostock et al., 1971).

It has been reported that block with 2 mM

E-Tdr is reversed by adding 2 mM E-dCdr (Xeros, 1962). We examined the effects of various concentrations of E-dCdr on nuclear DNA synthesis blocked with 2.5 mM E-Tdr and found that a certain range of concentrations of dCdr (0.01–0.02 mM) is optimal for reversing 2.5 mM E-Tdr block. On the other hand, we found that the efficiency of nuclear incorporation of ^{3}H-dCdr (2 μCi/ml, 2×10^{-4} mM) was highest in the presence of a certain range of concentrations of E-Tdr (2.5×10^{-1} mM$\sim2.5\times10^{-3}$ mM). These data showed that the ratio of the concentrations of these two nucleosides has a definite effect upon nuclear DNA synthesis. As reported before, 2.5 mM E-Tdr inhibits DNA synthesis of poxvirus to some extent (Mantani et al., 1970), but the inhibition was reversed by addition of 0.01–0.02 mM E-dCdr.

With regard to the relationship between the cell cycle and virus growth, we confirmed using E-Tdr-block and ^{3}H-dCdr that when viral DNA synthesis was initiated before the S phase, the infected cells did not enter the S phase, as already reported by Mantani and Kato (1970) using E-Tdr-block and ^{3}H-Tdr. This result agrees well with those obtained with Mengo virus, Newcastle disease virus (NDV) infection (Ensminger and Tamm, 1970), adenovirus infection (Hodge and Scharff, 1969) and herpes simplex virus infection (Cohen et al., 1971). However, cells infected with reovirus before the S phase enter the S phase (Ensminger and Tamm, 1969). Cellular protein synthesis was inhibited by Mengo, NDV infection (Ensminger and Tamm, 1970) and vaccinia virus infection (Moss, 1968). On the other hand, in reovirus infection there is no reduction in host cell protein synthesis (Ensminger and Tamm, 1969). In poxvirus infection, viral DNA was initiated at any phase of the cell cycle, except the M phase (Mantani et al., 1968; Mantani and Kato, 1971). Similar results were reported on herpes simplex virus infection (Cohen et al., 1971). However, on equine abortion virus infection, virus infection, viral DNA synthesis is dependent on the S phase of the cell cycle (Lawrence, 1971).

Autoradiography using ^{3}H-dCdr seems a useful method in systems in which ^{3}H-Tdr cannot be utilized.

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REFERENCES

Bootsma, D., L. Budke, and D. Vos. 1964. Studies on synchronous division of tissue culture cells initiated by excess thymidine. Exp. Cell Res. 33: 301–309.

Bostock, C. J., D. M. Prescott, and J. B. Kirkratirick. 1971. An evaluation of the double thymidine block for synchronizing mammalian cells at the Gl-S border. Exp. Cell Res. 68: 163–168.

Cohen, G. H., R. K. Vaughan., and W. C. Lawrence. 1971. Deoxyribonucleic acid synthesis in synchronized mammalian KB cells infected with Herpes simplex virus. J. Virol. 7: 783–791.

Ensminger, W. D., and I. Tamm. 1969. Cellular DNA and protein synthesis in Reovirus-infected L cells. Virology 39: 357–360.

Ensminger, W. D., and I. Tamm. 1970. Inhibition of synchronized cellular deoxyribonucleic acid synthesis during Newcastle Disease virus, Mengovirus, or Reovirus infection. J. Virol. 5: 672–676.

Grivell, A. R., and J. F. Jackson. 1968. Thymidine-kinase: Evidence for its absence from Neurospora Crossa and some other micro-organisms, and the relevence of this to the specific labeling of deoxyribonucleic acid. J. Gen. Microbiol. 54: 307–317.

Groyon, R. M., and A. J. Kniazeff. 1968. Vaccinia virus infection of synchronized pig kidney cells. J. Virol. 1: 1255–1264.

Hodge, L. D., and M. D. Scharff. 1969. Effect of adenovirus on host cell DNA synthesis in synchronized cells. Virology 37: 554–564.

Kato, S., M. Kurisu, and J. Kamahora. 1962. Effect of cytoplasmic DNA synthesis upon nuclear DNA synthesis in poxvirus-infected cells. Biken J. 5: 227–231.

Kato, S., M. Ogawa, and H. Miyamoto. 1964. Nucleocytoplasmic interaction in poxvirus-infected cells. I. Relationship between inclusion formation and DNA metabolism of the cells. Biken J. 7: 45–56.

Lawrence, W. C. 1971. Evidence for a relationship between Equine Abortion (Herpes) virus deoxyribonucleic acid synthesis and the S phase of the KB cell mitotic cycle. J. Virol. 7: 736–748.

Mantani, M., H. Miyamoto, and S. Kato. 1968. Effect of viral DNA synthesis of poxvirus upon nuclear DNA synthesis of synchronized FL cells. Biken J. 11: 71–90.

Mantani, M., Y. Sakaue, and S. Kato. 1970. Virus multiplication in culture cells synchronized by excess thymidine treatment. I. Effect of excess thymidine upon growth of poxvirus and chikungunya virus. Biken J. 13: 353–364

Mantani, M., and S. Kato. 1970. Virus multiplication in culture cells synchronized by excess thymidine treatment. II. Effect of viral DNA synthesis of Shope fibroma virus and cowpox virus upon nuclear DNA synthesis of synchronized FL cells. Biken J. 13: 365–376.

Mantani, M., and S. Kato. 1971. Virus multiplication in culture cells synchronized by excess thymidine treatment. III. Effect of viral DNA synthesis of cowpox virus upon the mitotic phase of FL cells. Biken J. 14: 297–310.

Mantani, M., and S. Kato. 1972. Lack of virus-induced cell surface antigen in metaphase-arrest cells infected with poxvirus. Biken J. 15: 105–109.

Morris, N. R., and G. A. Fischer. 1960. Studies concerning inhibition of the synthesis of deoxycytidine by phosphorylated derivatives of thymidine. Biochim. Biophys. Acta 42: 181–182.

Moss, B. 1968. Inhibition of HeLa cell protein synthesis by the vaccinia virion. J. Virol. 2: 1028–1037.

Reichard, P., Z. N. Canellakis, and F. S. Canellakis. 1960. Regulatory mechanisms in the synthesis of deoxyribonucleic acid in vitro. Biochim. Biophys. Acta 41: 558–559.

Rogers, A. W. 1967. Techniques of autoradiography. Elsevier Publishing Co., Amsterdam/London/New York.

Schaer, J. C., L. Ramseies, and R. Schindler. 1971. Studies on the division cycle of mammalian cells. Exp. Cell Res. 65: 17–22.

Studzinski, G. P., and W. C. Lambert. 1969. Thymidine as a synchronizing agent. I. Nucleic acid and protein formation in synchronous HeLa cultures treated with excess thymidine. J. Cell. Physiol. 73: 109–118.

Xeros, N. 1962. Deoxyriboside control and synchronization of mitosis. Nature 194: 682–683.