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Enzymatic Synthesis and Breakdown of Deoxyribonucleic Acid by Extracts of L Cells Infected with Vaccinia Virus

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SUMMARY

The enzymatic incorporation of radioactive thymidine into deoxyribonucleic acid (DNA) and the destruction of DNA have been studied in a crude enzyme system from uninfected L cells and those infected with vaccinia virus.

In infected cells, prior to the appearance of virus progeny both enzymatic activities increased and reached a maximum about 6 hours after infection. The maximal enzymatic activities for DNA synthesis and for DNA breakdown in extracts of infected cultures were respectively about 2.5 times and 7 times those of extracts of uninfected cultures. The former enzyme was found predominantly in the soluble fraction in the cytoplasm and the latter in the nuclear fraction.

Vaccinia virus completely inactivated by heat or UV treatment did not cause any change in the level of these enzymes. The results obtained indicate that the suppression of DNA synthesis in the nuclei of host cells after infection with these active or inactivated viruses is not directly associated with the alteration of these enzymatic activities.

INTRODUCTION

As shown in the previous report (Hanafusa, 1960) the nucleic acid metabolism is altered when L cell cultures are infected with active and inactivated forms of vaccinia virus. H^3-thymidine incorporation into nuclei is suppressed and labeled acid soluble compounds accumulate in the cytoplasm, while DNA synthesis in the cytoplasm was observed only in cells infected with active virus. The question arose from these results as to whether the levels of the enzymes participating in DNA metabolism are altered by viral infection.

An enzyme system capable of incorporating radioactive thymidine into DNA in extracts of *Escherichia coli* has been demonstrated by Kornberg *et al.* (Kornberg *et al.*, 1956; Lehman *et al.*, 1958) and the influence of bacteriophage infection on the synthetic pathway in host cells has also been investigated in detail, (Flaks *et al.*, 1959a, b; Somerville *et al.*, 1959; Keck *et al.*, 1960; Koerner *et al.*, 1959, 1960; Kornberg *et al.*, 1959). Similar enzyme systems were found in various tissues of rats (Bollum and Potter, 1958) calf thymus (Bollum, 1960) and ascites tumor cells (Smelie *et al.*, 1959, 1960). A polymerase was partially purified in some of these investigations.

The present paper is on changes in the activity of two enzyme systems catalysing the synthesis and the cleavage of DNA in cell-free extracts of L cells infected with active and inactivated vaccinia IHD virus.

MATERIALS AND METHODS

1. *Cells*

Monolayer cultures of Earle's L cells were used in this study. Methods of cell culture and cell counting were the same as those described in the previous paper (Hanafusa, 1960). The growth medium consisted of 95 per cent of La-Ye (Hanks' saline with 0.5 per cent lactalbumin hydrolysate and 0.1 per cent yeast extract) and 5 per cent calf serum.

2. *Virus*

Vaccinia IHD virus passaged in L cells was used. The virus stocks were prepared by sonic disruption of infected cells. After sonicating at 10 KC for 10 minutes, the suspension was centrifuged at 3,000 rpm for 10 minutes and then the supernatant virus fluid was kept in a deep-freeze. The method for inactivating the virus by heat and ultraviolet light was the same as described previously (Hanafusa, 1960). In this experiment completely inactivated viruses * used were prepared by heating virus samples at 56°C for 1 hour or irradiating them for 2 minutes with a UV lamp at a distance of 40 cm from the samples.

3. *Virus assay*

The virus titer was assayed by counting plaques on L cell plates. La-Ye solution was used for diluting the virus sample. The number of plaques was counted 2 - 3 days after plating.

4. *Determination of the DNA and protein content of cells*

The amount of DNA was determined colorimetrically according to the method of Burton (1956) using salmon sperm DNA as a standard. Protein was measured colorimetrically by the Folin-phenol reaction as modified by Lowry *et al.* (1951) using bovine albumin (Armour Laboratories, Fraction V) as a standard.

5. *DNA preparation*

The DNA preparation used as a primer was a high polymer preparation (California Corporation for Biochemical Research) from salmon sperm. In preliminary experiments, native and heated (100°C, 1 minute) DNA were used as primers, but no difference was found between them. Therefore, native DNA was used throughout these experiments.

The labeled DNA used for DNase assay, was obtained from L cells labeled with H^3-thymidine for 4 - 5 hours. After labeling, cells were harvested in 5 ml of Hanks' saline and 2.0 ml of 20 per cent trichloroacetic acid were added in the cold, and the suspension was centrifuged. The resulting precipitate was dissolved in 1.0 ml of cold 1 N KOH and then treated with 2 ml of water, 0.2 ml of 6 N HCl and 1 ml of 20 per cent trichloroacetic acid. The precipitate was collected by centrifugation and dissolved in 0.2 ml of 1 N KOH. 0.2 N HCl was added dropwise to a pH of 7.5 and then 0.05 M Tris buffer was added to make the volume up to 5.0 ml. 0.1 ml of such a preparation contained 0.71 mg of DNA and 1.11×10^5 cpm of radioactivity.

6. *Isotope*

Tritium labeled thymidine (4.0 c/m mole, The Radiochemical center, England) was diluted with

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* The following abbreviations are used in this paper; HIV: heat inactivated vaccinia, UIV: UV-inactivated vaccinia virus.

distilled water to appropriate concentrations. Radioactivity was measured as described previously (Hanafusa, 1960).

7. *Preparation of cell extracts*

L cell cultures were infected with 1.0 ml of virus samples and incubated at 37°C for 2 hours. Then the cultures were washed with La-Ye, overlaid with 8 ml of growth medium and incubated at 37°C. Control cultures were inoculated with 1.0 ml of growth medium. The uninfected and infected cultures were chilled at various times and washed twice with Hanks' saline. The washings were completely removed. Cells were harvested in 0.05 M Tris-HCl buffer (pH 8.0) using a rubber policeman. Usually 3 ml of buffer were used for 4 bottles of cell cultures each bottle containing 8×10^6 cells. The cell suspension was subjected to sonic oscillation at 10 KC for 5 minutes in a polyethylene centrifuge tube and then centrifuged at 12,000 $\times$ g for 20 minutes. The supernatant solution containing about 2 mg protein per ml was used as the enzyme solution without further purification. All the above procedures were carried out at 0 - 4°C. The enzyme solution was kept in an ice bath, and no loss of enzyme activity was found during a three day storage period.

In some cases cultured cells were harvested and washed by centrifugation. However reproducible data were not obtained by this method, because, as shown by spectrophotometry, a considerable amount of acid soluble material leaked out of the cells into the washing media.

8. *Fractionation of subcellular components*

Homogenates were prepared by disrupting L cells in an all glass homogenizer in 2 ml of buffered sucrose solution containing 0.25 M sucrose, 0.013 M Tris-HCl (pH 8.0) and 0.005 M KCl. Nuclei were prepared by centrifuging the whole homogenate for 10 minutes at 600 $\times$ g, and subjecting the sediment to one further re-homogenization and re-sedimentation. (fraction a) Under the microscope, the homogenized sample appeared to contain a least some whole cells. The homogenized preparations were separated into the following fractions by means of centrifugation and sonic oscillation.

a. whole nuclei
b. a sonicated "fraction a" (sonicated at 10 KC for 5 minutes, and then centrifuged at 12,000 $\times$ g for 20 minutes)
c. the whole supernatant after centrifugation at 600 $\times$ g
d. the supernatant after centrifugation of "fraction c" at 12,000 $\times$ g
e. a sonicated "fraction c", (sonicated at 10 KC for 5 minutes, and then centrifuged at 12,000 $\times$ g for 20 minutes)

All the above procedures were done at 0-4°C.

9. *Standard assay procedure of enzymatic activities*

a) *Assay of enzymatic synthesis of DNA*

This assay measured the incorporation of H^3-thymidine into an acid-insoluble product. The reaction mixture (0.80 ml) contained 28 μ moles of Tris-HCl (pH 8.0); 4.0 μ moles of $MgCl_2$; 2.0 μ moles of ATP ; 1 μc of H^3-thymidine; 250 μg of salmon sperm DNA and 0.20 ml of cell extract containing 0.4 mg of protein. After incubation at 37°C for 60 minutes, the tube was chilled, and 1.0 ml of water and 0.8 ml of 20 per cent trichloroacetic acid were added. After centrifugation at 3,000 rpm for 5 minutes, the precipitate was dissolved in 0.5 ml of 1 N KOH. The DNA was reprecipitated by the addition of 1.5 ml of water, 0.1 ml of 6 N HCl and 0.8 ml of 20 per cent trichloroacetic acid, and centrifuged. The precipitate was dissolved in 0.2 ml of 1 N KOH and diluted to a suitable volume. 0.2 ml aliquots of this solution were pipetted into planchets and their radioactivity was measured.

b) *Assay of DNase*

The assay measured the release of radioactive acid-soluble fragments from DNA. The incubation mixture (0.40 ml) contained 4.0 μ moles of $MgCl_2$; 14 μ moles of Tris-HCl (pH 8.0); 0.1 ml of

H^3-labeled DNA obtained from L cells and 0.2 ml of cell extract containing 0.4 ml of protein. After incubation of the mixture at 37°C for 40 minutes, 0.1 ml of carrier DNA (2.5 mg per ml) and cold 1 N perchloric acid were added. After removal of the precipitate, the supernatant solution was neutralized with 0.8 ml of 1 N KOH and kept at 0°C for 30 minutes. Then the precipitate was removed by centrifugation. 0.2 ml aliquots of the supernatant solution were plated, and the radioactivity was determined.

RESULTS

1. *One-step growth curve for vaccinia virus in L cells*

To obtain a one-step growth curve of vaccinia virus in monolayers of L cells, monolayers containing on the average 8.6×10^6 cells per culture were infected with 1.2×10^8 PFU/ml of vaccinia IHD virus. Two hours after infection, the inoculum was removed and the cells were washed 5 times with La-Ye and overlaid with 8 ml of growth medium. Infected cultures were kept at 37°C. At various time intervals after infection, cultures were chilled and after removing the supernatant solution, the cells were harvested in 2 ml of fresh growth medium in a rubber stopped cellulose acetate tube* with a rubber policeman. The cell suspension

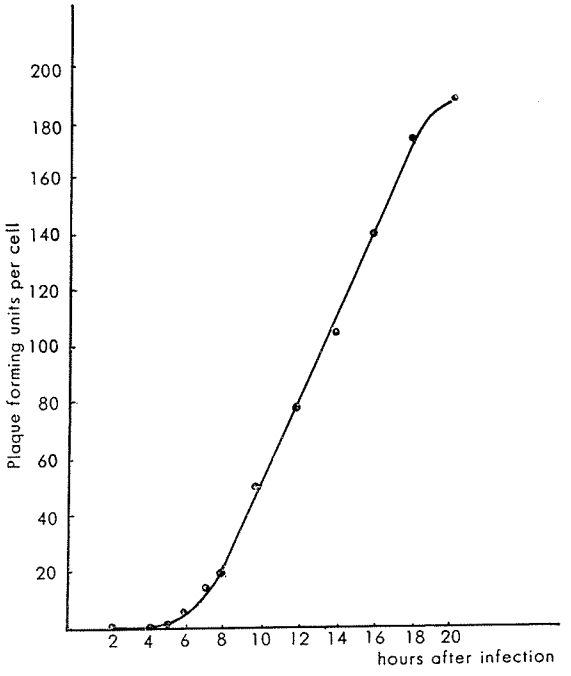


Fig. 1. Growth of the IHD strain of vaccinia virus in L cells. Monolayers containing 8.6×10^6 cells/culture were infected with 1.2×10^8 PFU/ml of the virus. The curve shows the rate of increase in the infectious units in cells.

* The tubes used were those for the "40" rotor of a Spinco L preparatory centrifuge.

was sonicated in a 10 KC sonic oscillator for 10 minutes and then centrifuged at 3,000 rpm for 10 minutes. The number of plaque forming units in the resulting supernatant solution was determined.

A one step growth curve (Fig. 1) indicated that the first increase in intracellular virus occurred at 6 hours and that the yield was about 200 plaque-forming units per infected cell at 20 hours.

2. *Enzymatic synthesis of DNA by the cell extract*

a) *Properties of the enzyme system*

The enzymatic activity for incorporating thymidine into DNA was measured with extracts of uninfected L cells and with those infected with vaccinia virus, by the standard assay method. Thymidine incorporation at 37°C as a function of the incubation period and the relation between the protein concentration and thymidine incorporation are shown in Figs. 2 and 3. The reaction proceeded linearly for 80 minutes, and was proportional to the amount of enzyme with cell

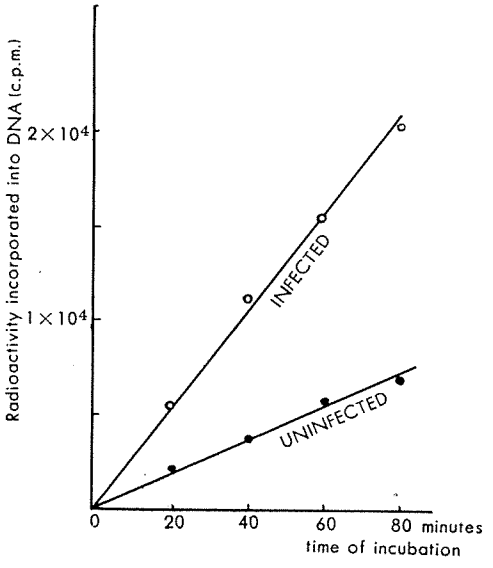


Fig. 2. Incorporation of thymidine into DNA as a function of time. Reaction mixtures as described in Materials and Methods were incubated at 37°C for the indicated periods

extracts containing up to 0.6 mg of protein. The presence of primer DNA and Mg^{++} was indispensable for this reaction, as shown in Table 1, ATP seemed to be required as a phosphorus donor. In this experiment, the precursors of DNA depend upon substances pooled in the cells. One important question is whether variations in the nucleotide pool in the specimens affects the assay of enzymatic activity. To test this, extracts of infected cells were heated at 100°C for 1 minute and centrifuged. To the reaction mixture used in the standard procedure was

added 0.2 ml aliquots of the supernatant of the heated extracts. The incorporation of thymidine was not affected by the above procedure.

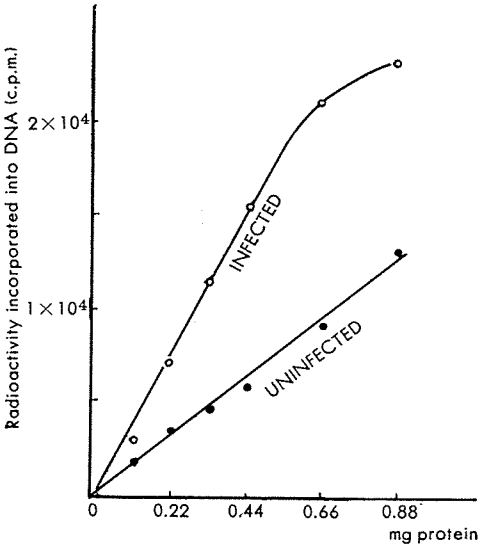


Fig. 3. Relation between thymidine incorporation into DNA and protein concentration. Varying amounts of cell extract were added to the reaction mixture which was then incubated for 60 minutes.

Table 1. Requirements for Thymidine Incorporation

Extract	DNA added	Mg^{++} added	ATP added	H^3-thymidine incorporated into DNA
Uninfected	0 μg	4.0 μg	2.0 μmoles	< 1000 cpm/mg protein
	50	4.0	2.0	3500
	125	4.0	2.0	11100
	250	4.0	2.0	14000
	250	4.0	0.5	8100
	250	4.0	1.0	11100
	250	0	2.0	3800
Infected	0	4.0	2.0	7700
	50	4.0	2.0	43000
	125	4.0	2.0	41200
	250	4.0	2.0	40000
	250	4.0	0.5	31200
	250	4.0	1.0	37000
	250	0	2.0	2300

The reaction mixture contained the following; H^3-thymidine, 1 μc (4μc/mμ mole); Tris-buffer, pH 8.0, 28 μ moles; 0.10 ml of cell extract (2.0 mg protein/ml) and DNA, Mg^{++}, ATP as indicated. Final volume, 1.0 ml. Incubated 60 minutes at 37°C.

b) *Enzymatic synthesis of DNA after infection*

As shown in Fig. 4, incorporation of thymidine into DNA began to increase immediately after infection and at 6 hours after infection it reached a maximum level which was about 2.5 times that in the uninfected cultures. Subsequently it remained unaltered or decreased slightly.

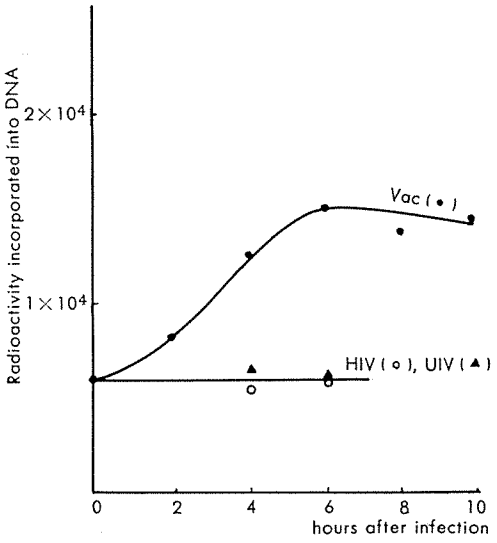


Fig. 4. The level of DNA synthesizing enzyme after infection with vaccinia virus, HIV and UIV. The assay method was described in the Materials and Methods.

Several enzymatic steps may be involved in the incorporation of thymidine into DNA and the results obtained show the change in the rate of over-all incorporation. When the acid soluble fraction from cells infected with UV-irradiated vaccinia virus and labeled with H^3-thymidine, was used as substrate for the reaction, the increase in activity was very slight. The main components of this acid soluble fraction seemed to be deoxyribonucleoside triphosphates (Hanafusa, 1960). This fact suggests that the level of the "polymerase" catalysing the conversion of deoxyribonucleoside triphosphates into DNA does not change much after infection. This supposition was supported by another experiment. A reaction mixture containing extracts of the infected cells was incubated without primer DNA for 40 minutes and then heated at 100°C for 1 minute, according to the method of Bollum and Potter (1958). The supernatant of the resulting mixture was used as substrate in reaction mixtures which were then incubated for 40 minutes. The thymidine incorporation was almost equal in uninfected and infected cell extracts.

Fig. 4 also indicated that infection with neither HIV nor UIV resulted in a change in the enzyme level in the host cells.

c) *Cellular localization of the enzyme activity*

Uninfected or infected cells were homogenized and the nuclei and cytoplasm separated. The enzymatic activities in the five fractions separated (a-e, in section 8 of Materials and Methods) were determined. Maximal specific activity was found in the supernatant fraction (fraction d), as reported already (Bollum and Potter, 1958), and minimal activity in the nuclear fraction (fraction a) of either uninfected or infected cells. (Table 2)

Table 2. Cellular Localization of the Enzymatic System Incorporating Thymidine into DNA

Fraction*	Enzymatic activity (radioactivity incorporated into DNA)	Protein	Specific enzymatic activity
Uninfected	cpm	μg	cpm/mg protein
a	348	206	1.69×10^3
b	176	115	1.53×10^3
c	11700	776	15.1×10^3
d	13000	612	21.2×10^3
e	11600	676	17.2×10^3
Infected			
a	394	244	1.62×10^3
b	194	138	1.41×10^3
c	24100	708	34.1×10^3
d	29400	548	53.7×10^3
e	26200	572	45.9×10^3

* The fractionation procedure is described in section 8) of Materials and Methods. The reaction mixtures were standard except that the various fractions were used as enzyme preparations

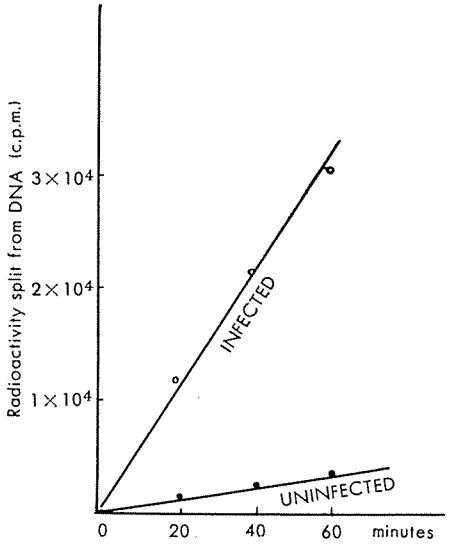
3. *The level of DNase*

a) *Properties of DNase*

The activity of DNase was determined by a standard assay method. Breakdown of DNA as a function of time and the relation between protein concentration and breakdown are shown in Figs. 5 and 6. From these results, about 0.4 mg of protein and a 40 minute reaction period were adopted as suitable conditions for later experiments.

Released acid-soluble fragments could not be measured spectrophotometrically because of the presence of a large amount of acid soluble nucleotide.

The DNases of extracts from uninfected or infected cells hydrolysed both host-DNA and viral DNA. Labeled viral DNA was prepared from cells infected with vaccinia virus in the following manner. Infected cells were labeled with H^3-thymidine for 2 - 6 hours after infection, and then the cells were treated twice with 0.1 M citric acid. The extracted cytoplasmic DNA was precipitated with trichloroacetic acid, and the labeled viral DNA was obtained by procedures similar



Fig, 5. Breakdown of DNA as a function of time. Reaction mixture as described in the Materials and Methods was incubated at 37° C for the indicated periods.

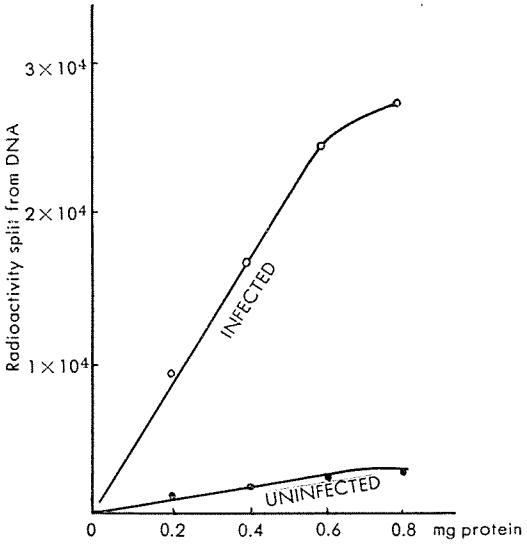


Fig. 6. Relation between breakdown of DNA and protein concentration. Varying amounts of cell extract were added to the reaction mixture and incubated for 40 minutes.

to those used for labeled host DNA. Host DNA and viral DNA were hydrolysed at almost equal rates by extracts of either uninfected or infected cells. Dilution of substrate DNA with a nonlabeled salmon sperm DNA caused a decrease in the rate of release of radioactivity to a degree proportional to the dilution. These

results indicate that the substrate specificity of the DNase in both uninfected and infected cells is very low.

b) *The level of DNase after infection*

Fig. 7 shows the change in the DNase activity after infection. The activity

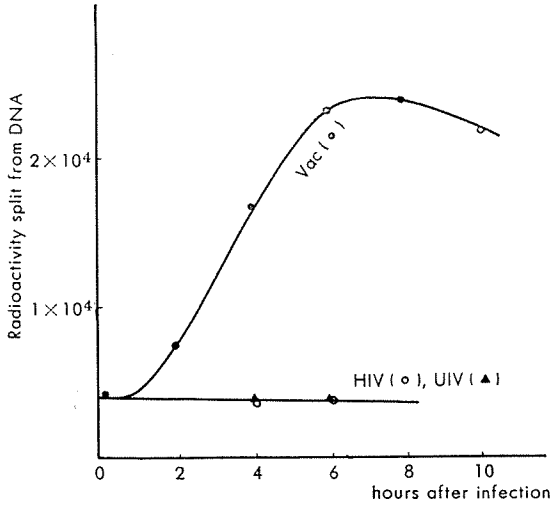


Fig. 7. The level of DNase after infection with vaccinia virus, HIV and UIV. The assay method was as described in Materials and Methods.

had increased approximately 7 times 6 hours after infection and later the level decreased slightly. At its maximal level, the enzyme released about 21 per cent of the radioactivity of DNA into the acid soluble fraction. The level of DNase was the same in extracts of cells infected with HIV and with UIV.

It was found by Kozloff (1953) that the DNase in extracts of T_2 infected *E. coli* was inhibited by extracts of uninfected *E. coli*. This inhibitory action was due to some RNase sensitive substance. Kozloff considered the apparent increase in DNase after phage infection to be a result of destruction of this inhibitor after infection. In the present study, however, no inhibitory action against DNase was found in extracts of uninfected cells.

c) *Cellular localization of DNase activity*

This was studied in the same manner as the synthesis of DNA. As summarized in Table 3, in contrast with the synthesizing system, DNase was most active in the nuclear fraction, with about half as much activity in the cytoplasmic fraction. Therefore it seems likely that this enzyme is located in the nuclei rather than in the cytoplasm.

Table 3. Cellular Localization of the DNase

Fraction*	Enzymatic activity (radioactivity released from DNA)	Protein	Specific enzymatic activity
Uninfected	cpm	μg	cpm/mg protein
a	3080	206	15.0×10^3
b	1600	115	14.0×10^3
c	6420	776	8.26×10^3
d	5200	612	8.50×10^3
e	5440	676	8.05×10^3
Infected			
a	14200	244	58.3×10^3
b	11700	138	84.8×10^3
c	29800	708	42.0×10^3
d	24600	548	44.9×10^3
e	27000	572	47.1×10^3

* The fractionatian procedure is described in section 8) of Materials and Methods. The reaction mixtures were standard except that the various fractions were used as enzyme preparations.

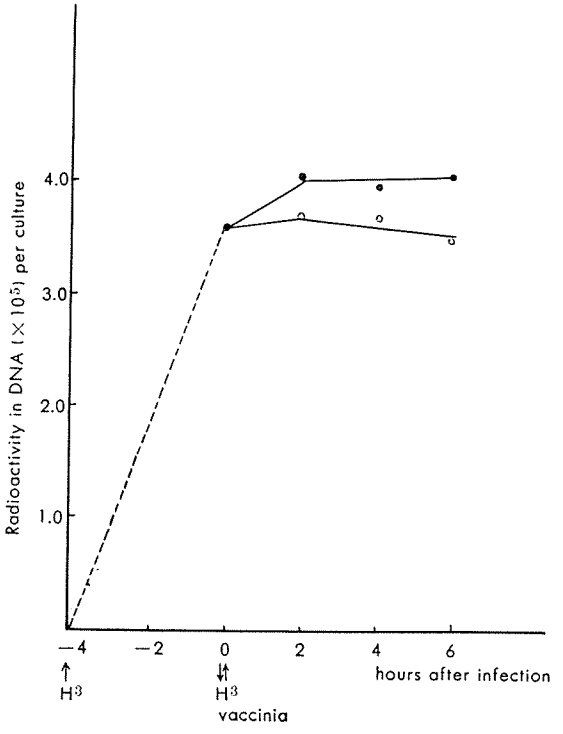


Fig. 8. Effect of infection with vaccinia virus on DNA in nuclei of host cells were labeled with H^3-thymidine for 4 hours and then infected with vaccinia virus for 2 hours. The DNA in nuclei was extracted from the cultures at 0 (just before infection), 2, 4, 6 hours after infection. Uninfected cells (•), Infected cells (○).

4. *Turn over rate of cellular DNA*

The increase in DNase activity after vaccinal infection is quite rapid. The increase in DNase activity may be associated in some way with the mechanism of synthesis of viral DNA. It may produce precursors of viral DNA by breakdown of the host DNA. Therefore, the rate of turn over of nuclear DNA in infected cells was determined.

Monolayers of L cells were cultured in Medium 199 containing H^3-thymidine (2 μc/5 ml/culture) for 4 hours, and then washed with Hanks' saline. These labeled cultures were infected with vaccinia virus, and after a 2 hours' adsorption period the inoculum was replaced by 8.0 ml of growth medium. In the uninfected control cultures, 1.0 ml of growth medium was used as the inoculum. Cells were chilled at various times, washed twice with Hanks' saline, and then treated with 0.1 M citric acid at 37°C as described in an earlier report (Hanafusa, 1960), DNA was precipitated from the nuclear fraction and the radioactivity of the nuclear DNA was measured. As shown in Fig. 8, there was only a slight difference in rate of turn over of DNA in uninfected and infected cultures. This indcates that host DNA once synthesized is rather stable and it is not utilized during multiplication of vaccinia virus.

DISCUSSION

The present studies demonstrate that there is a definite increase in two enzyme systems following viral infection, one is system which incorporates thymidine into DNA, and the other is one that breaks down DNA.

The system for synthesizing DNA studied in this work seems to catalyse an over-all reaction and it is not possible to draw any conclusion about the nature of the various enzymes involved. However, from the assay method, the requirement for DNA and Mg^{++} for this synthetic reaction was found to be similar to that of systems from other sources (Lehman *et al.*, 1958; Bollum, 1960). The rate of thymidine incorporation was found to be increased in extracts of infected cells. The increase in rate of this enzyme system was unexpectedly high and by the time viral progeny had begun to maturate the activity had already reached its maximum. Preliminary experiments suggest that the level of "polymerase" was unchanged after infection, but the validity of this fact will be checked by further studies using four deoxyribonucleoside triphosphates as substrates.

Although DNase activity was also increased after infection, the effect of DNase upon the assay of the synthetic enzyme was not considered. If corrections were made for this, the increase in the activity of the latter enzyme would be greater.

As mentioned already, there was very great suppression of thymidine incorporation into the nuclei of UIV-infected cells. Nevertheless, the activity of the DNA synthesizing enzyme system in extracts of UIV-infected cells was the same

as that of uninfected cultures. Possible explanations for these facts are as follows. (1) In cells infected with UIV, the DNA synthesizing enzyme is masked by some inhibitory substance, which is released by disruption of the cells. (2) The UV-damaged DNA of UIV caused some hindrance of transfer of "information" from cellular DNA to the rest of the cell, resulting the suppression of DNA synthesis in nuclei. (3) Some mechanism of transportation of the DNA synthesizing enzyme to the site where it functions is inhibited by infection. (4) Precursors for nuclear DNA synthesis are directed to viral DNA synthesis after infection, though the latter synthesis is abortive in L cells infected with UIV. The true explanation must await results of further studies.

An unexpected finding was that there was a large increase in DNase after infection. In addition, the change of the level of this enzyme after infection was almost proportional to that of the synthesizing enzyme, and 6 hours after infection maximal activity was observed. These results imply that the DNase level is dependent of the replication process of this virus. However, studies on the rate of turn-over of nuclear DNA using radioactive thymidine showed that the host DNA was not in a position to be utilized for the synthesis of viral DNA. Furthermore, the level of DNase was not altered in UIV-infected cells. Obviously, DNase is not responsible for the suppression of thymidine incorporation into nuclear DNA in cells which is induced by infection with active virus, UIV and HIV.

It has been found that infection of *E. coli* B with phage T_2 caused a rapid increase in the DNase activity of cell extracts (Cohen, 1949; Kunkee and Pardee, 1956). This increase may be due to the removal of DNase inhibition rather than to the synthesis of enzyme (Kozloff, 1953). On the other hand, in T_5 phage infected *E. coli,* the DNA content of the culture fell rapidly during the first few minutes after infection and then increased at a rapid rate (Pfefferkorn and Amos, 1958; Crawford, 1959). However, it was pointed out by Crawford (1959) that the increase in DNase may not be related to the breakdown of host DNA. This breakdown may be due to a highly specific DNase which represents only a small fraction of the total DNase activity. However there is no evidence on this point yet.

Since the DNase found in extracts of infected L cells was not inhibited by the addition of extracts of normal cells, the increase in this enzyme activity represents a net synthesis of enzyme protein after infection. This might be a kind of cellular response to abnormal DNA synthesis induced by viral infection.

ACKNOWLEDGEMENTS

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