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Alteration of Nucleic Acid Metabolism of Host Cells by Active and Inactivated Forms of Vaccinia Virus

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SUMMARY

The rate of incorporation of tritiated thymidine and cytidine into cellular fractions of cultures infected with active vaccinia virus and with heat or UV inactivated virus was studied. In general, those infections suppressed nucleoside incorporation into nuclear DNA and accumulated labeled compounds into the acid soluble fraction of the cytoplasm. The effect on DNA synthesis was greatest with UV inactivated virus. DNA synthesis in the cytoplasm was observed only in cells infected with active virus and was fastest 4 hours after infection. Heat treated virus induced almost the same alteration in nucleic acid metabolism in the cells as active virus excepting that after infection with it labeled precursor could not be converted to cytoplasmic DNA. These results seem to support the hypothesis that both inactivated forms of vaccinia virus can induce some reaction within infected cells and that killing of host cells by them is due to a disturbance in the host cell metabolism.

Chromatographic analysis of labeled, acid soluble compounds accumulating in cultures infected with UV inactivated virus, showed that thymidine triphosphate was a major component.

INTRODUCTION

Vaccinia virus contains DNA as a structural nucleic acid component and infection of host cells results in synthesis of new DNA in their cytoplasm. Many morphological and biochemical investigations have been made on the mechanism of multiplication of this group of viruses. Recently biochemical analysis of the multiplication of this virus has been mainly directed to the synthesis of nucleic acids induced by active virus (Joklik and Rodrick, 1959; Magee and Sagik, 1959; Salzman, 1960; Magee *et al.*, 1960; Kato *et al.*, 1960; Cairns, 1960). From these studies the following conclusions have been obtained. (1) the nucleic acid content of the cells was not greatly affected by infection, (2) viral DNA was actually synthesized and accumulated in the cytoplasm of infected cells, (3) its synthesis occurs rather early; it is completed before maturation of the progeny virus takes place. These remarks will serve to interpret the *de novo* synthesis of viral DNA in the cytoplasm. However, no investigation has been made on the effect of any inactivated form of this virus on nucleic acid metabolism.

Flaks *et al.* (1959) indicated in their studies on deoxycytidylate hydroxymethylase induced by T_2 phage infection that UV-inactivated phage also retained its capacity to induce formation of this enzyme. It was demonstrated that multiple infection

with either heat- or UV-inactivated virus caused the death of host cells (Hanafusa, 1960b), and that heat inactivated virus could be reactivated by superinfection with a heterologous active pox virus and interfere with infection by a homologous virus (Hanafusa *et al.*, 1959a, b; Hanafusa, 1960a; Joklik *et al.*, 1960a, b) These findings suggest that inactivated virus might induce some reaction within infected cells. Thus, if death of cells was due to some disturbance in the host cell metabolism, infection by these inactivated forms might in some manner affect the pattern of incorporation of nucleosides. Therefore, the alteration of nucleic acid metabolism on infection with active and inactivated virus was followed using thymidine H^3 and cytidine H^3 as isotopic tracers.

MATERIALS AND METHODS

1. *Cells*

Monolayer cultures of Earle's L cells were grown in Hanks' saline containing 0.5 per cent lact-albumin hydrolysate, 0.1 per cent yeast extract and 0.5 per cent calf serum.

The number of cells was determined by counting the nuclei in a haemocytometer. 4 ml of 0.1 M citric acid were added to the culture before counting and the cells incubated at 37°C for 1 hour. The nuclei after centrifugation at 600 *g* for 10 minutes were resuspended in 1 ml of 0.1 M citric acid containing 0.01 per cent gentian violet and 0.25 per cent methyl-cellulose (4000 centipoise) and after shaking put into a haemocytometer. For quantitative experiments about 5×10^6 cells per culture were used.

2. *Virus*

The IHD strain of vaccinia virus passaged in L cells was used. The virus sample was obtained by sonic disruption of infected cells suspended in growth medium. A Kubota 200 watt, 10 KC magneto-strictive oscillator for 10 minutes was used for sonic disruption. After diluting to a concentration of 2×10^8 PFU/ml, the resulting virus preparations were stored in a deep-freeze.

Heat inactivated material was obtained by heating the virus stock in an ampoule at 56°C for one hour. Material was UV-irradiated using a 15 watt germicidal tube (Toshiba Electrics). The virus stock to be inactivated was exposed to UV light for 2 minutes in 2 ml aliquots in a petridish (87 mm I.D.) at a distance of 40 cm from the lamp (Hanafusa, 1960b).

3. *Infecting and labeling of cells*

Monolayer cultures, from which the fluid had been removed, were inoculated with 1.0 ml of active or inactivated viruses. The virus sample was allowed to adsorb at 37°C for 2 hours; it was then replaced by 5 ml of growth medium. Under these conditions, approximately 80 per cent of the inoculated virus was adsorbed. The inoculated cultures were incubated at 37°C.

Both tritium labeled thymidine (4.0 c/m mole) (The Radiochemical Centre, England) and cytidine (1.0 c/m mole) (Schwarz Bioresearch, Inc., New York) were dissolved in M 199 (Difco Laboratories) which does not contain these nucleosides. At intervals after infection, varying with the experiment, various concentrations of thymidine H^3 or cytidine H^3 was added to the culture. After incubating for two hours to permit labeling, the cultures were washed five times with saline before further treatment.

4. *Fractionation of infected cells*

In experiments in which the incorporation of labeled nucleosides was followed, cells were fractionated into nuclei and cytoplasm using citric acid solution. Although this method does not produce good results for many chemical estimations, it has been recommended by Dounce (1955) for the isola-

tion of nuclei for nucleic acid determinations. Onto the monolayer cells which had been washed five times with saline, 4 ml of 0.1 M citric acid solution was added and the bottles were incubated at 37°C for one hour. The nuclei were separated from the harvested suspension by centrifugation at 600 *g* for 10 minutes. The sediment was again treated with citric acid at 37°C for 20 minutes. The combined supernatant fluids were called the "cytoplasmic fraction."

Acid soluble compounds and nucleic acids in both "nuclear" and "cytoplasmic" fractions were separated by cold perchloric acid treatment, according to the method of Schneider (1945). If any further separation of nucleic acids was necessary, the acid insoluble residue was dissolved in 1 N KOH and kept at 37°C overnight. By acidification of this alkaline hydrolysate with hydrochloric acid and trichloroacetic acid, the DNA was precipitated while the RNA remained in the supernatant (Schmidt and Tannhauser, 1945).

For measurements of radioactivity in precipitates, such as whole nuclei, acid insoluble nucleic acids and DNA, material was dissolved in a small quantity of 1 N KOH and diluted with water.

When the DNA content of cells was measured colorimetrically, the DNA fraction of cells was obtained by the method of Schneider (1945). Cells were precipitated with 0.2 N perchloric acid, washed twice with cold 0.2 N perchloric acid, once with cold ethanol, and then extracted with hot ethanol-ether (3: 1). Nucleic acids in this defatted precipitate were extracted twice with 0.5 N perchloric acid at 90°C for 20 minutes.

5. *Measurement of radioactivity*

0.2 ml aliquots of each sample were plated onto three planchets and the radioactivity was measured with a windowless gasflow counter fitted with an automatic sample changer (Nuclear Chicago Corp.). Conditions approximating to infinite thinness were usually employed; however when this was not possible, a suitable correction was made for self-absorption from specially constructed reference curves. A sufficient number of counts were recorded to keep the counting error below 5 per cent.

6. *Determination of the DNA content of cells*

The DNA content of cells was determined colorimetrically according to the method of Burton (1956) using a standard sample of deoxyribose.

7. *Chromatography of acid soluble compounds*

For chromatography of acid soluble compounds, 5 ml of an acid soluble extract, which had been neutralized and deionized with KOH, was introduced onto a column (0.9 × 12 cm) of Dowex-1 (X-8, 200 to 400 mesh) resin. Adsorbed nucleotides were fractionated by extended gradient elution chromatography with the "formic acid system" of Hurlbert *et al.* (1954). After washing the column with 10 ml of water, they were eluted by successively introducing the following eluents into a mixing flask previously filled with 250 ml of water; 400 ml of 4N formic acid, 400 ml of 4N formic acid-0.2 M ammonium formate, 200 ml of 4N formic acid-0.4 M ammonium formate and 400 ml of 4N formic acid-0.8 M ammonium formate. Four ml fractions were collected at a flow rate of 20-24 ml per hour. The extinction of each tube was measured at 260 mμ and 275 mμ in a Beckman DU spectrophotometer. An aliquot was taken from each tube for radioactivity measurements.

Although this method of fractionation of acid soluble compounds gives systematic and reproducible results, it was desired to shorten the time of elution and to sharpen the peaks, especially of nucleosides triphosphate in the analysis of acid soluble, labeled compounds. For this purpose, the above procedure was slightly modified as follows. The above four eluents were replaced by 800 ml of 1.0 M ammonium formate in 4 N formic acid, which was introduced into the mixing flask previously containing 250 ml of water. In this way, thymidine triphosphate could be eluted in tubes 110 to 140 (Fig. 7). All chromatographic experiments were carried out at 2°C.

8. *Abbreviations*

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

inactivated vaccinia, T-5′-P: thymidine -5′- phosphate, TDP: thymidine diphosphate, TTP: thymidine triphosphate, ATP: adenosine triphosphate, PFU: plaque forming units.

RESULTS

For biochemical analysis of the nucleic acid metabolism of infected cells, some basic biological information on the system is a prerequisite. To establish definite experimental conditions, various examinations were performed on the metabolic state of the host cells, on the multiplicity of inactivated virus sufficient to cause complete death of cells and on the rate of uptake of labeled nucleosides by host cells.

Salzman (1959) has reported that the cellular nucleic acid content fluctuates during growth of monolayer cultures of mammalian cells. Therefore the DNA content of cells after subculture was examined under the conditions used. As seen in Fig. 1, cells began to proliferate about 24 hours after subculture, but the amount of DNA in the culture subsequently continued to increase steadily. Excess DNA seemed to accumulate during the lag phase and its cellular content became stationary after two

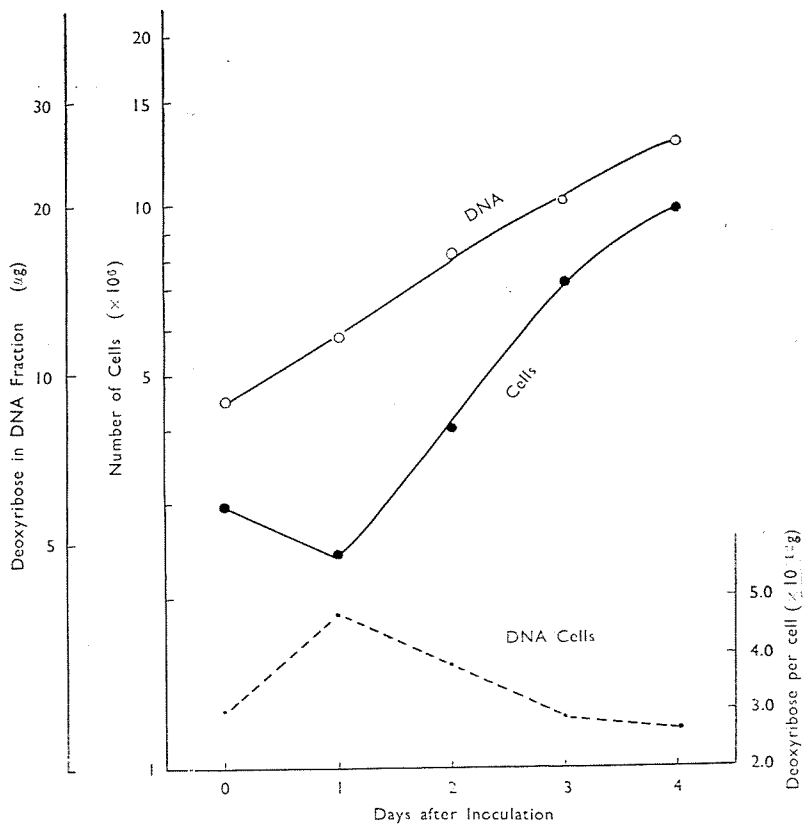

Fig. 1. Change of DNA content of L cell monolayers after subculture.

to three days. From the above results, which agreed with Salzman's report, two day old cultures were used throughout this study.

It has been shown that death of L cells by UV- and heat inactivated vaccinia virus occurs only with multiple infections (Hanafusa, 1960b). The growth curves of cells infected with active and inactivated vaccinia of various multiplicities are shown in Fig. 2.

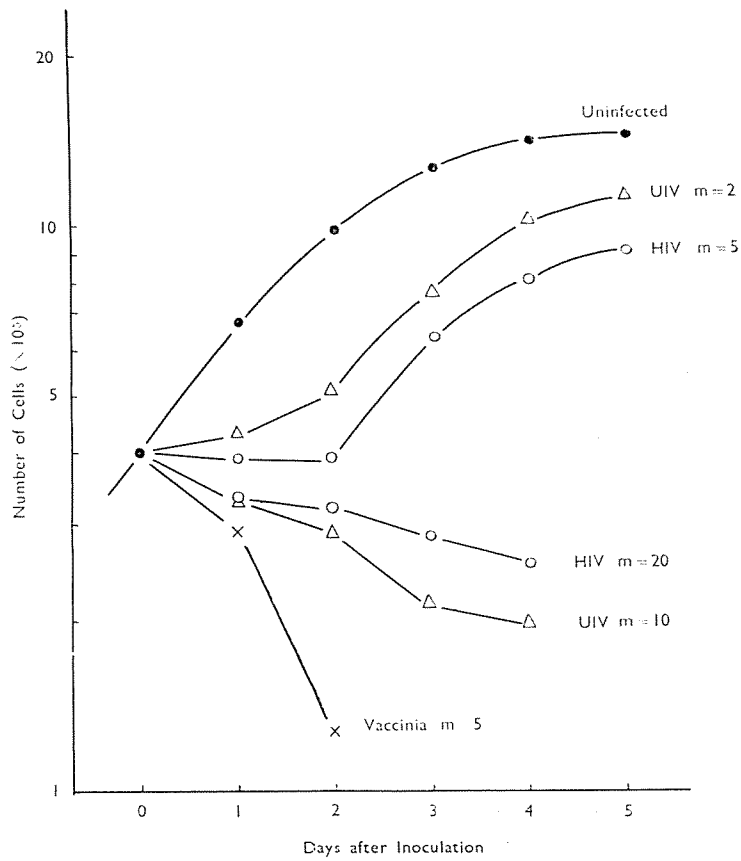

Fig. 2. Effect of infection with active or UV- and heat-inactivated vaccinia virus on multiplication of L cells in monolayer culture. The number of cells was estimated by counting the nuclei.
m: multiplicity of infection

Thus it is necessary to use inactivated virus with a multiplicity of more than 20 to cause complete killing of cells. In all experiments with active virus, UV- and heat-inactivated virus a multiplicity of 40 was used in this study.

Since mammalian cells are known to utilize exogenous nucleosides (Hurlbert *et al.*, 1954; Friedkin *et al.*, 1956; Friedkin and Wood, 1956), labeled compounds of

high specific activity were dissolved in the nutrient medium without carrier so that they would be predominantly incorporated into the cells. Fig. 3 shows that thymidine H^3 was very rapidly incorporated into uninfected L cells and the radioactivity in the nuclei reached a maxmum level after 6 hours incubation. Since during the first 2 hours, incorporation was practically linear, an incubation period of 2 hours was adopted.

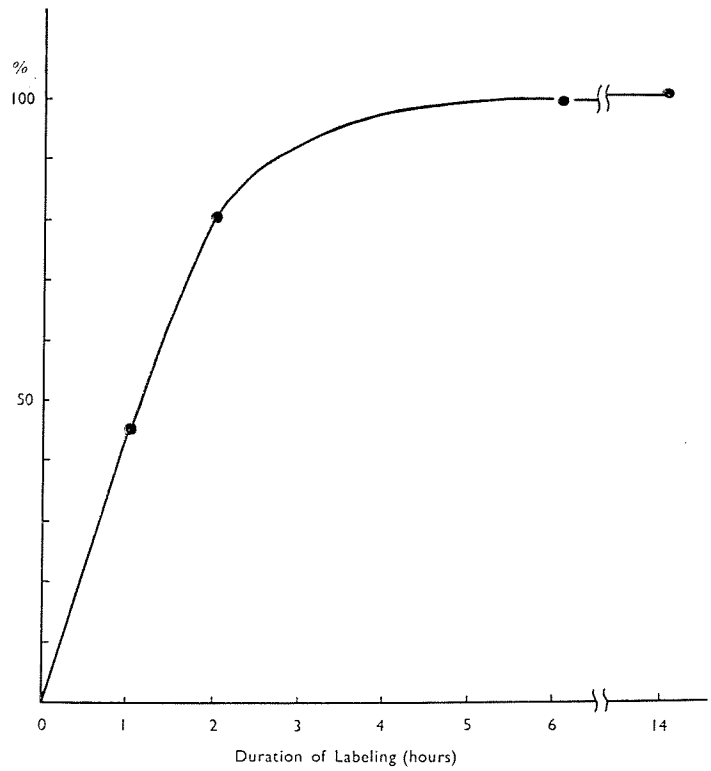

Fig. 3. The effect of the duration of labeling on the incorporation of thymidine H^3 by uninfected L cells.
Radioactivity incorporated into nuclei during the indicated intervals was presented as a percentage of the 14 hours value.

1. *Uptake of thymidine H^3 by whole cells*

Thymidine H^3 was added to uninfected and infected cells 4 and 6 hours after infection and after 2 hour's incubation the incorporation of radioactivity into the cells was measured (Table 1).

The radioactivity of uninfected cells and cells infected with active virus or HIV was almost equal at both times after infection. There was a slight difference between uninfected and infected cells at 4 hours, which, however had almost disappeared by

6 hours. This result is not in accord with that reported by Magee *et al.* (1960), who found a stimulation of thymidine uptake on infection. A most striking fact was the suppression of nucleoside uptake after infection with UV inactivated vaccinia virus; 6 hours after infection the rate of uptake of radioactivity by these cells had dropped to about third of that by uninfected control cells.

Table 1. Incorporation of thymidine H^3 into whole cells.

Inoculated virus	Radioactivity in whole cells (cpm per 5×10^6 cells)	
	4 hours after infection	6 hours after infection
Uninfected	51.0×10^4	47.0×10^4
HIV	46.3	48.6
UIV	28.6	16.1
Vaccinia	54.0	45.5

Cultures were exposed to thymidine H^3 (2.0μ c/5 ml/bottle) for 2 hours from the indicated times after infection.

2. *Incorporation of thymidine H^3 into cellular fractions of infected cells*

According to radioautographic studies on cultured cells infected with vaccinia virus (Kato *et al.*, 1960; Cairns, 1960; Magee *et al.*, 1960), after infection, nuclear DNA synthesis by the host cells was suppressed and viral DNA was produced in the cytoplasm. Indeed, the DNA content of the cells, as estimated colorimetrically, was unchanged after infection (Unpublished results). Therefore, it seemed interesting to study which cellular fraction the labeled nucleosides were incorporated into and to what extent they were utilized in polymer synthesis.

Thymidine H^3 (2.0 μc/5 ml/bottle) was added to infected cultures at various times after infection and after 2 hours the nuclei and cytoplasm fractionated and each fraction was further separated into acid soluble and acid insoluble compounds. As, in a preliminary experiment, thymidine was found to be incorporated only into the DNA, and not into the RNA of the acid insoluble fraction further fractionation of acid insoluble fraction was omitted.

As shown in Fig. 4, it is evident that, compared with uninfected cultures, the rate of incorporation into the nuclei of cells infected with HIV, UIV or active virus was generally low and the degree of suppression of incorporation increased with time. As expected from experiments with whole cells, there was marked suppression in UIV infected nuclei. On the other hand, there was much incorporation into the cytoplasms of all these infected cells, but very little into the cytoplasm of uninfected cells. The labeled compounds found in the cytoplasmic fractions were acid soluble, and were perhaps low molecular compounds, except in cells infected with active virus. In cultures infected with active virus, labeled thymidine was also incorporated into the cytoplasmic DNA fraction, the rate being maximal 4-6 hours after infection, later the radioactivity increased in the acid soluble components. These results agree with

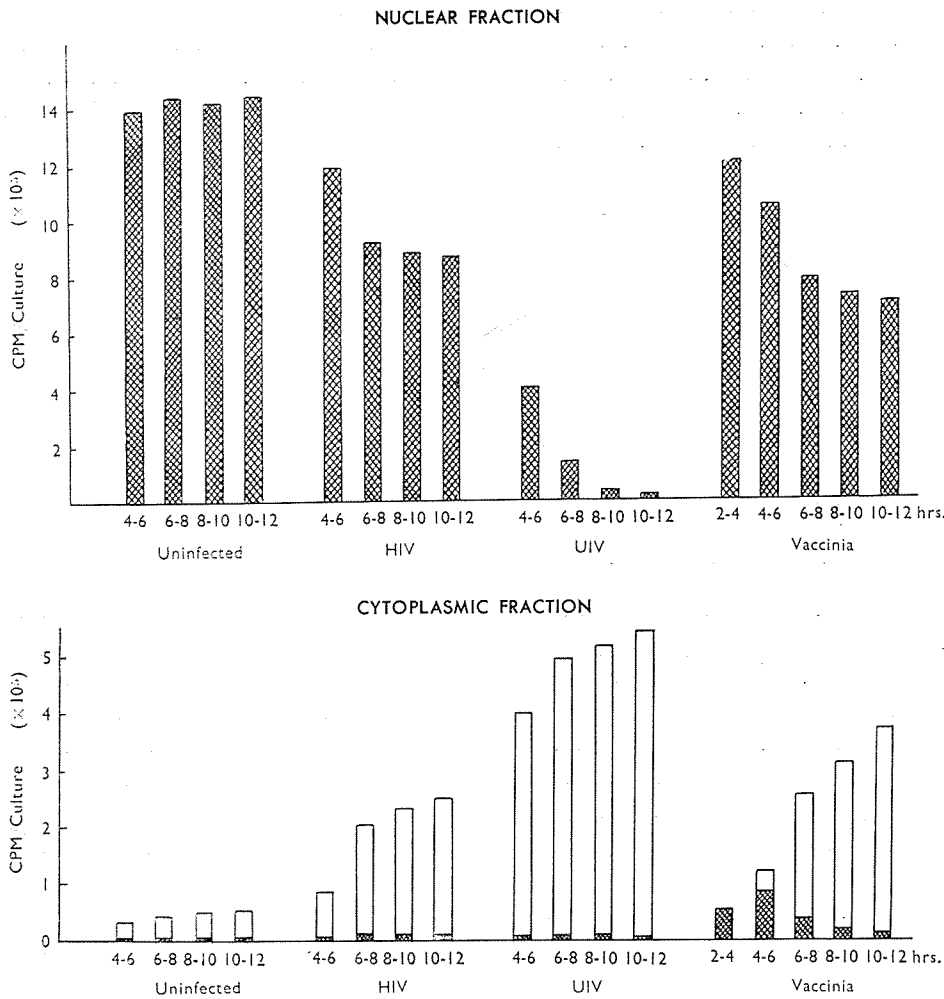

Fig. 4. Kinetics of thymidine H^3 incorporation into cultures infected with HIV, UIV and active vaccinia virus and into uninfected cultures. The cultures were labeled with thymidine H^3 at the time intervals after infection indicated in the figure. The height of the bars shows the total radioactivity in each nuclear and cytoplasmic fraction. The amount of radioactivity in the DNA fraction is represented by the crosshatched area of the bars, and in the acid soluble fraction by the empty areas.

those of Magee *et al.* (1960) and of Salzman (1960), who both indicated that synthesis of viral DNA was very active 4-6 hours after infection and that it was complete prior to production of mature progeny virus.

3. *Incorporation of cytidine H^3 into cellular fractions*

It has been found that cytidine is easily incorporated into both the RNA and

DNA of mammalian cells (Reichard and Estborn, 1951; Feinendegen, 1960). In the present experiments, incorporation of cytidine H^3 was used as an index of the changes in RNA metabolism in infected cells and as a confirmation of results with thymidine H^3. Cytidine H^3 (1.0 μc/5 ml/bottle) was used for labeling and the labeled cells were treated in the same manner as in the above experiments with thymidine H^3. In

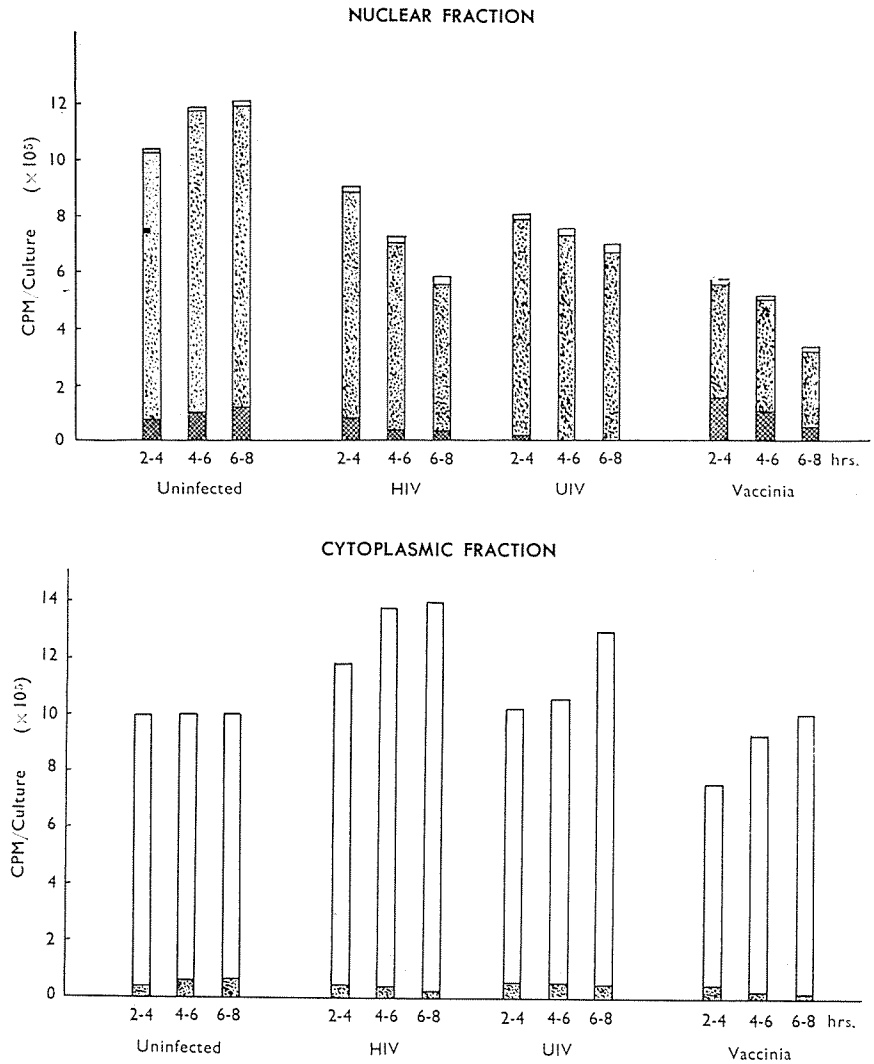

Fig. 5. The kinetics of cytidine H^3 incorporation into the cultures infected with HIV, UIV and active vaccinia virus and into uninfected cultures. The cultures were labeled with cytidine H^3 at the time intervals after infection indicated in the figure. The height of the bars shows the total radioactivity in each nuclear and cytoplasmic fraction. Amounts of radioactivity in the RNA fraction are represented by the stippled areas of the bars, in the DNA fraction by the crosshatched areas and in the acid soluble fraction by the empty areas.

this case, the acid insoluble residue was separated into RNA and DNA fractions.

The incorporation patterns of uninfected and infected cultures are illustrated in Fig. 5. Cytidine incorporation was inhibited in the nuclear fraction by infection with active virus, HIV or UIV. However, incorporation of radioactivity into nuclear DNA showed the same tendency as thymidine incorporation; drastic suppression was found only in UIV infected nuclei. In the cytoplasmic fraction, incorporation of cytidine H^3 was stimulated in HIV and UIV infected cells and slightly suppressed in cells infected with active virus but it accumulated in all of them with time. As no detectable radioactivity was found in the cytoplasmic DNA fraction even in cells infected with active virus, it may be that cytidine was mainly incorporated into the nuclear RNA during 2 hours labeling period, its reduction to deoxycytidylic compounds not being so rapid, and that it was not directly utilized for the synthesis of viral DNA. Experiments with both thymidine H^3 and cytidine H^3 indicated that inhibition of nucleic acid synthesis by UIV-infection was more profound in the DNA than the RNA fractions.

4. *Chromatography of acid soluble compounds in UIV infected cells*

From the above experiments using labeled nucleosides, it was concluded that infection of UV inactivated virus led to a marked suppression of nuclear DNA formation and accumulation of acid soluble compounds in the cytoplasm. To determine at which stage DNA synthesis was interrupted, the acid soluble compounds were fractionated by ion exchange chromatography.

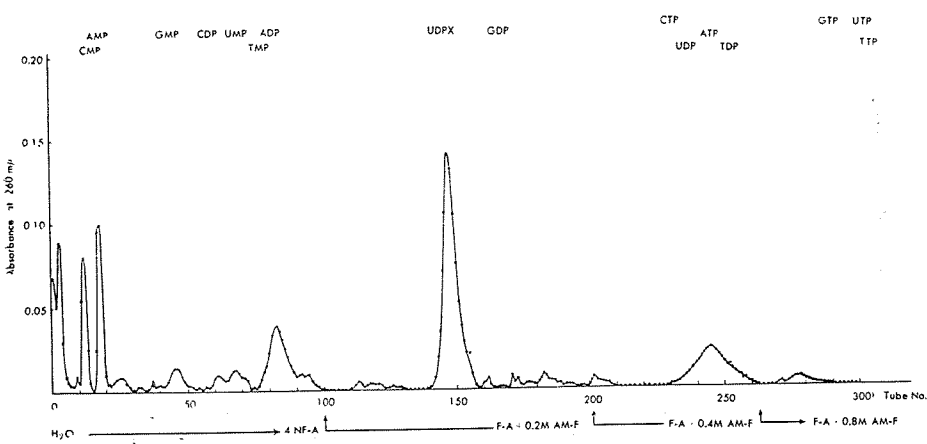

Fig. 6. Chromatography of the acid-soluble fraction of uninfected L cells on a Dowex-1 column (formate) by gradient elution with the formic acid system. The optical density at 260 mμ is plotted for each chromatographic fraction. In the upper portion of the figure, the location of various known nucleotides is shown.

In preliminary experiments, the acid soluble fraction of uninfected L cells was chromatographed with the standard "formic acid system". The chromatographic

pattern of a representative experiment (Fig. 6) shows that various nucleotides and their phosphorylated derivatives are eluted at similar effluent volumes to those reported by Hurlbert *et al.* (1954). The highest peak, appearing in tubes 140 to 155, was considered to represent uridylic acid derivatives as judged from the ratio of absorption at 275 mμ to 260 mμ. (In Fig. 6 it is tentatively named UDPX). The pattern of acid soluble compounds was not affected by active infection.

As negligible amounts of acid soluble compounds were found in the nuclei of UIV infected cultures, they were obtained from whole cells exposed to 4.0 μc of thymidine H^3 for 6 to 8 hours after infection with UIV. Labeled cells were harvested and homogenized twice with 0.5 N perchloric acid in the cold. After centrifugation, the supernatant solution was neutralized with 5 N KOH, and $KClO_4$ which precipitated was removed. The samples thus obtained were introduced onto a Dowex-1 column and eluted with the "formic acid system" of Hurlbert *et al.* (1950), or by the modified method described in MATERIALS AND METHODS. As shown in Fig. 7, three main peaks of radioactivity corresponding to T-5′-P, TDP and TTP were

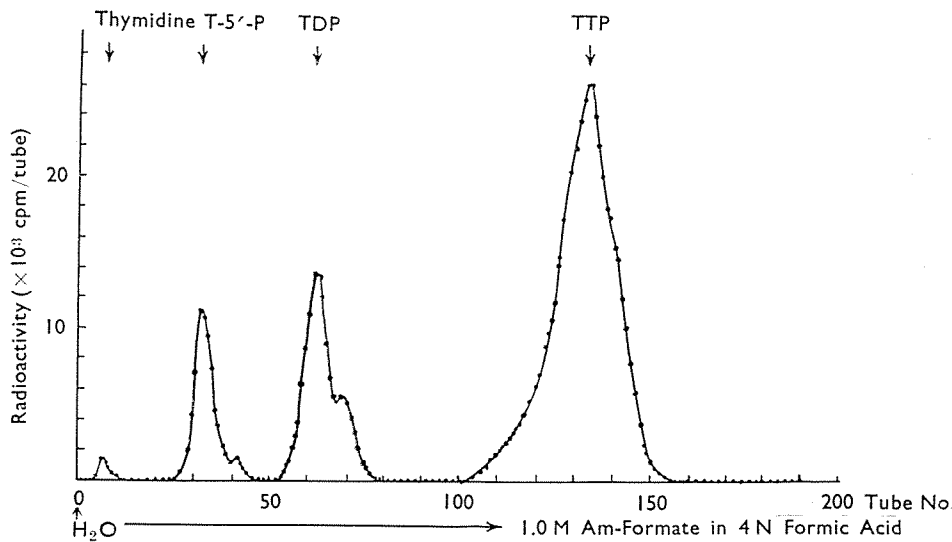

Fig. 7. Chromatography of the acid-soluble fraction of cells infected with UIV and labeled with thymidine H^3 by the modified formic acid system. The radioactivity is plotted for each chromatographic fractions. Each component was identified after rechromatography using the standard formic acid system.

obtained and in other tubes no radioactivity was detected. Recovery of radioactivity was as high as 94 per cent. The first peak, which is considered to be derived from thymidine not adsorbed onto the column, was very small and the ratio of radioactivity in the peaks of T-5′-P, TDP and TTP was calculated as 10.4 : 19.2 : 70 5.

From this experiment it seems that the incorporated nucleoside could be pho-

phorylated in UIV-infected cells and that the interruption of DNA synthesis induced by UIV may be mainly at the step of polymerization of the nucleotide triphosphate. However, the problem of whether the accumulated TTP was synthesized as a precursor of viral DNA by induction of the UIV-genome or whether it resulted from an inhibition of conversion to host nuclear DNA cannot yet be answered.

DISCUSSION

Results on the alteration in cellular metabolism induced by active virus infection in these experiments essentially confirm the work of other investigators.

As seen in Fig. 4, suppression of nuclear DNA synthesis and accumulation of acid soluble compounds was marked, especially about 6 hours after infection with active virus. This alteration in the incorporation pattern, however, might be due to degeration of the host cells rather than to synthesis of viral DNA, since inactivated viruses produced the same effects in the host cells, as discussed below.

The incorporation pattern in cells infected with HIV was very similar to that in cells infected with active virus except that synthesis of cytoplasmic DNA was found only in the latter. In the preceding papers some biological properties of heat inactivated virus have been described. Heat treated virus could be reactivated by superinfection with other active pox viruses and could interfere with the replication of homologous active virus. The heat stable DNA of this virus may be the causative agent of these phenomena. These facts suggest that heat treated virus retains the capacity for inducing some initial reactions necessary for virus synthesis within the cells. Joklik *et al.* (1960c) reported that vaccinia virus which has been partially inactivated by nitrogen mustard could reactivated heat inactivated rabbit-pox virus. They concluded that the mechanism of this reactivation was by the rescue of protein parts of the heated virus by another virus. Although it is not clear whether treatment with nitrogen-mustard causes a loss of activity only in the DNA of the virus, this work is compatible with our hypothesis.

In cultures infected with UV-irradiated virus, DNA synthesis was strongly inhibited and nucleoside triphosphate accumulated. Hakala (1959) and Littlefield and Gould (1960) showed the toxic effect of nucleoside analogue, 5-bromo-deoxyuridine on mammalian cells. As small a dose as 10 μg/ml could arrest cell division and cause degeneration of the cells. They showed that this compound was incorporated into the DNA molecule in place of the thymine residue and the resulting abnormal DNA caused death of the cells. This suggests the possibility that the effect of UV-inactivated virus might be due to similar compounds, produced in the virus sample by UV-irradiation. The supernatant solution of sonicated L cells was irradiated with UV light and inoculated into L cells under the condition used for UV-irradiated virus. No difference in the rate of thymidine H^3 uptake between the untreated and the inoculated cells could be detected. Therefore, the above possibility can be excluded

and it seems likely that the irradiated virus itself, which has some UV lesions on its DNA, causes a metabolic disturbance.

As already mentioned, multiple infection with either UV or heat inactivated virus, causes death of the host cells. The effect of these inactivated viruses on nucleic acid metabolism becomes obvious within 4 hours after inoculation. This indicates that the disturbance of nucleic acid metabolism is closely related to their process of cell-killing.

Radioautography of cultures infected and exposed to thymidine H^3 gave similar results on biochemical analysis; in cultures infected with HIV or UIV, the amount of silver found in the nuclei was less than that in uninfected nuclei and no condensed silver grain areas in the cytoplasm, which were striking in cultures infected with active virus, could be found.

The following remarks should be taken into consideration before any conclusions are drawn from these results. The first concerns with the procedure used for fractionation of cells. The citrate method was used to separate the nuclei from the cytoplasm in this study, but it has been recorded that some proteins are extracted from the nuclei by this procedure (Dounce, 1955; Kay *et al.*, 1956). Therefore, some acid soluble compounds from the cytoplasmic fraction might come from the nuclear fraction. Second, the reactions involved in nucleic acid synthesis in the host cells have not yet been determined. For example, thymidylic acid might be synthesized by methylation of deoxyuridylic acid (Friedkin and Kornberg, 1957; Maley and Maley, 1959). If so, results obtained using labeled nucleosides as tracers would not represent the metabolic activity of the normal pathway. Finally, in the present experiments, both active and inactivated viruses were infected at a multiplicity of 40, which was enough to cause complete death of the cells. It may be that the higher the multiplicity of infection, the more rapidly does degeneration of the cells take place. Experiments with lower multiplicities of infection seem to be important to examine the above possibility.

Studies on these problems are now in progress.

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