CHAPTER IV

RESULTS

1. Titration of Stock Virus

Plaque forming assay was used in the measurement of the infectivity of stock HSV-2. Total of 20 bottles of HSV-2 infected cells were pooled. After sonication of the infected cells, the supernate was collected and divided into small aliquots and kept at -70 °C as stock virus. It was found that the titer of the stock HSV-2 was 5.0×10^6 PFU/mL as shown in Figure 1.

2. Cell Toxicity Assay

HeLa cells at the density ranging from $5 - 30 \times 10^3$ cells per well were grown in 96-well microtiter plates in the presence of increasing concentration of acyclovir, and the viable cell counted by tryphan blue exclusion assay was carried out after a 37 °C incubation for 24 h. It was found that acyclovir concentrations ranging from 0-10 ug/mL had no significant effect on viability of HeLa cells (Figure 2). The density of cell at 2.0×10^4 cells/wells was chosen in the further works because at this density the cells gave a confluent monolayer.

3. Plaque Reduction Assay

The effects of acyclovir on the yield of HSV-2 at

various multiplicities of infection or at different time-courses of infection was determined by plaque reduction assay.

3.1 Inhibitory Effect of Acyclovir on HSV-2 Multiplication at Various Multiplicities of Infection

HeLa cells were allowed to infect with HSV-2 at MOI of 2.5, 5, 10 and 25 in the presence of 0, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL of acyclovir. After which the inhibitory effect of acyclovir on the virus yield was tested by the plaque reduction assay and the results was summerized in Table 1. The yield of virus was partially inhibited at 0.05 ug/mL of acyclovir and completely inhibited at 0.1 to 4.0 ug/mL of acyclovir. In the presence of 0.05 ug/mL of acyclovir the yield of virus was reduced approximately 10 times as compared to the control at the MOI of 2.5 while it was reduced 20 times at the MOI of 5. Results obtained in this study suggested the HSV-2 at the MOI of 10 was the optimal MOI for the assay and therefore was chosen in the study for the effect of acyclovir at different time courses of infection.

3.2 Inhibitory Effect of Acyclovir on HSV-2 Multiplication at Different Time-courses of Infection

HeLa cell monolayer was infected with HSV-2 at MOI 10. After allowing of infection for 1.5, 6.0 or 12.0 h, acyclovir at concentrations ranging from 0-4 ug/mL

were added. Thereafter, they were frozen and thawed three times. The viral content in the cultures was then titrated by the plaque assay. It was found that the yield of the virus was completely inhibited at the concentrations of 0.05 ug/mL and 1.0 ug/mL of acyclovir after infection for 1.5 and 6.0 h, respectively (Table 2). However, at 12.0 h after infection, acyclovir could not compltely inhibit the yield of the virus even with the use of acyclovir at the concentration as high as 4.0 ug/mL.

4. Inhibitory Effect of Acyclovir on DNA Synthesis of the HSV-2 Infected Cells

The DNA synthesis of HSV-2 infected cells in the presence of acyclovir was determined by incorporation of ^{3}H-thymidine into the cells. In our preliminary experiment showed that in the presence of 0.2 ug/mL of acyclovir de novo DNA synthesis was inhibited to 56, 74, and 66 % as compared to the control cultures in the use of 0.2, 0.5 and 1.0 uCi/mL of ^{3}H-TdR, respectively (Table 3). Similarly, the inhibition of ^{3}H-TdR incorporation was observed in the cultures of HSV-2 infected cells treated with 1.0 ug/mL of acyclovir. It was appeared that the amount of 0.5 uCi/well of ^{3}H-TdR was the optimal concentration for the assay (Table 3) and therefore was used through out the experiment.

4.1 Inhibitory Effect of Acyclovir on DNA Synthesis at Various Multiplicities of Infection

HeLa cells were infected with HSV-2 at MOI of 2.5, 5, 10 and 25. After a viral adsorption for 1.5 h, the cells were treated with acyclovir concentrations ranging from 0-4 ug/mL and then pulse-labelled with 0.5 uCi ^{3}H-TdR/well. It was found that in the presence of 0.2 ug/mL of acyclovir DNA synthesis was inhibited to 17 % as compared to the control at MOI of 5 while it was inhibited up to 30 % and 33 % at MOI of 10 and 25 respectively (Table 4). Similarly, the inhibition of ^{3}H-TdR incorporation was observed in the cultures of HSV-2 infected cell treated with 0.5 - 4.0 ug/mL of acyclovir. It was found that the infection at MOI of 10 was appeared to be the appropriate MOI for the assay. However, the inhibition at MOI of 2.5 could not compared to the control because at this MOI the virus concentration was too low to achieve the complete infection, resulting in inconsistency results.

4.2 Inhibitory Effect of Acyclovir on DNA Synthesis at Different Time-courses of Infection

HeLa cells were infected with HSV-2 at MOI of 10, the infected cells were then exposed to acyclovir 0-4 ug/mL for 1.5, 6.0, and 12.0 h after infection and pulse-labelled with ^{3}H-thymidine. As shown in Table 5, HSV-2 DNA synthesis at 1.5 h was significantly inhibited at the concentration of equal to or more than 0.2 ug/mL acyclovir ($P < .05$). The significant inhibition at 6.0 h and 12.0 h were observed at concentrations of acyclovir lower than at 1.5 h. It was also found that the viral DNA

synthesis was greatly inhibited by acyclovir treatment after infection for 6.0 h. In this time course, the presence of 0.05 ug/mL of acyclovir DNA synthesis was inhibited to 28 % as compared to the control and the inhibition was increased up to 75% in the culture of HSV-2 infected cell treated with 4.0 ug/mL of acyclovir. However, at 12 h after infection, the viral DNA synthesis was inhibited to 60 % as compared to the control at 4.0 ug/mL of acyclovir.

5. Inhibitory Effect of Acyclovir on Polypeptides Synthesis of the HSV-2 Infected Cells

The inhibitory effect of acyclovir on the synthesis of HSV-2 polypeptides was studied by SDS-PAGE and Western blotting technique. After an electrotransfer of the bands from SDS-PAGE onto a nitrocellulose, the remaining proteins in the gel which were not completely transferred were detected by staining in 0.25 % Coommassie brilliant blue R. It was appeared that only trace amount of high-molecular-weight-proteins were demonstrated (Figure 3).

The antigenic polypeptides of HSV-2 on the nitrocellulose were demonstrated by an immunoblotting technique. Our previous experiments found that the suitable concentration of protein appeared to be 3.0 ug of protein per lane and the good resolution of discreted bands occured by using the combination of rabbit immunoglobulin to HSV-2 at the dilution of 1:20 and

peroxidase conjugated swine antirabbit at the dilution of 1:50. Therefore, the concentration of protein and the dilutions of antiserum and conjugate were used through out the immunoblotting experiments.

5.1 Inhibitory Effect on HSV-2 Polypeptide Synthesis at 1.5 Hours Post Infection

The reaction of rabbit immunoglobulin to HSV-2 polypeptieds by the immunoblotting was shown in Figure 4. Lane A represented polypeptides of HSV-2 untreated with acyclovir, reacted with HSV-2 rabbit immunoglobulins. There were a number of antigenic bands with molecular weights of 20-130 Kd, including 5 major HSV-2 glycoprotein antigens : glycoprotein gB (110-130), gG (92), gC (80), gE (70-75), gD (60), and LMW glycoproteins. Immunoblotting of mock-infected cells, prepared in the same manner as infected cells,did not react to the rabbit immunoglobulins (data not shown). Lane B-E represented polypeptides of HSV-2 treated with acyclovir at concentrations of 0.2, 0.5, 1.0, 2.0 and 3.0 ug/mL, respectively. The pattern of polypeptides by immunoblotting of HSV-2 treated with acyclovir was similar to that of HSV-2 untreated with acyclovir but the overall reaction was proportionally decreased as the concentrations of acyclovir increased.

5.2 Inhibitory Effect on HSV-2 Polypeptide Synthesis at 6.0 Hours Post Infection

The reaction of HSV-2 polypeptides treated or untreated with acyclovir at 6.0 h post infection with HSV-2

rabbit immunoglobulins were shown in Figure 5. Lane A showed the pattern of the immunoblotting of untreated HSV-2 polypeptides which 5 major HSV-2 glycoprotein antigens and LMW glycoproteins were observed. This pattern was similar to that of at 1.5 h after infection. Lane B-G showed HSV-2 polypeptides treated with acyclovir at concentrations of 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL, respectively. There was overall similarity in the pattern of HSV-2 polypeptides treated and untreated with acyclovir, however, the intensity of bands was proportionally decreased. In addition, HSV-2 polypeptides were almost completely inhibited when the culture was treated with 3.0 ug/mL of acyclovir.

5.3 Inhibitory Effect on HSV-2 Polypeptide Synthesis at 12.0 Hours Post Infection

A representative patterns of HSV-2 polypeptides in the cultures treated and untreated with acyclovir at 12.0 h post infection were shown in Figure 6. Lane A showed acyclovir untreated HSV-2 polypeptides reacted with rabbit immunoglobulins. This lane demonstrated polypeptides having molecular weights of 20-130 Kd which were glycoproteins gB, gG, gC, gE, gD and LMW glycoproteins according to published data of molecular weight of HSV-2 polypeptides. Lane B-G showed HSV-2 polypeptides in the cultures treated with acyclovir at the concentrations of 0.2, 0.5, 1.0, 2.0, 3.0, and 4.0 ug/mL, respectively. Lane H showed mock infected cells prepared

in the same manner as infected cells. Although the pattern of HSV-2 polypeptides in the treated and untreated groups were not striking differences, however, the degrees of intensity of bands of HSV-2 polypeptides in the acyclovir treated cultures were proportionally lesser than that of the untreated control. Particularly in the culture treated with 3.0 ug/mL of acyclovir,the HSV-2 polypeptides synthesis were almost completely inhibited, except the molecular weight at approximately 20 Kd polypeptide.

6. Comparison of the Inhibitory of Effect of Pure Chemical Acyclovir and Intravenous Acyclovir

6.1 Inhibitory Effect of Pure Chemical and Intravenous Acyclovir on HSV-2 Multiplication by Plaque Reduction Assay

The effect of pure chemical acyclovir and intravenous acyclovir on the yield of virus at 6.0 h post infection with HSV-2 were compared in the range of 0-4.0 ug/mL. The results in table 6 showed that both forms of acyclovir had significantly inhibitory effect upon the yield of the virus. It is noted that at concentration of 0.2 ug/mL of pure chemical acyclovir could completely inhibit the yield of the virus whereas the intravenous acyclovir completely inhibited the yield of the virus at the concentration of 1.0 ug/mL.

6.2 Inhibitory Effect of Pure Chemical and Intravenous Acyclovir on DNA Synthesis of HSV-2 by ^{3}H-Thymidine Incorporation

Comparison of the inhibitory effect of pure chemical acyclovir and intravenou acyclovir was carried out at 6.0 h post infections (Table 7). Similar results obtained were showing that both acyclovir at the concentration as low as 0.05 ug/mL could significantly inhibit HSV-2 DNA synthesis in the cultures treated with acyclovir as compared to the controls ($p < 0.05$). The variations in the mean of count per minute of each forms of acyclovir were noted, however, the percentages of the mean of count per minute when compared with its control were proportionally decreased as increased in the concentrations of both forms of acyclovir. In the presence of acyclovir at the concentration of 4.0 ug/mL, DNA synthesis was inhibited as much as 71% and 75% as compared to the controls in the use of pure chemical and intravenous acyclovir, respectively. It is, therefore, the DNA synthesis of HSV-infected cell was found to be sensitive to the inhibition by both forms of acyclovir.

6.3 Inhibitory Effect of Pure Chemical Acyclovir on Polypeptide Synthesis of HSV-2 Infected Cells

The reactions of HSV-2 polypeptides in the cultures treated or untreated with pure chemical acyclovir at 6.0 h post infection reacted with rabbit immunoglobulin against HSV-2 were shown in Figure 5. Lane A revealed HSV-2 polypeptides having molecular weight ranging from 20-130 Kd in the acyclovir-untreated HSV-infected culture reacted with the rabbit immunoglobulins. The immunoblotting

strip also showed clearly visible 5 major HSV-2 glycoproteins and LMW glycoproteins. Treatment with acyclovir ranging from 0.2-4.0 ug/mL reduced the synthesis of HSV-2 polypeptides (Lane B-G), especially with the concentration of 3.0 ug/mL of acyclovir almost completely diminished the synthesis of HSV-2 polypeptides (Lane F). Control culture of mock infected cells shown in Lane H demonstrated no visible band can be found.

Table 1 In vitro inhibitory effect of acyclovir on HSV-2 multiplication at various multiplicities of infection.

MOI	Virus yield : No. of PFU/mL								
	Doses of acyclovir (ug/mL)								
	0	0.05	0.1	0.2	0.5	1	2	3	4
2.5	$2.4x10^4$	$2x10^3$	0	0	0	0	0	0	0
5	$2.0x10^4$	$1x10^3$	0	0	0	0	0	0	0
10	$2.2x10^{04}$	0	0	0	0	0	0	0	0
25	$6.7x10^4$	0	0	0	0	0	0	0	0

Table 2 In vitro inhibitory effect of acyclovir on HSV-2 multiplication at different time courses of infection

Hour post-infection (MOI=10)	Virus yield : No. of PFU/mL								
	Doses of acyclovir (ug/mL)								
	0	0.05	0.1	0.2	0.5	1.0	2.0	3.0	4.0
1.5	$2.2x10^4$	0	0	0	0	0	0	0	0
6.0	$3.5x10^4$	$6x10^3$	$1x10^3$	$4x10^3$	$2x10^3$	0	0	0	0
12.0	$3.0x10^4$	$7x10^3$	$2x10^3$	$3x10^3$	$6x10^3$	$6x10^3$	$5x10^3$	$4x10^3$	$5x10^3$

Table 3 Establishment of optimal condition for ^{3}H-thymidine incorporation

Amount of ^{3}H-TdR (uCi/well)	Mean of count per minute (CPM)±SE		
	HSV-2	HSV-2+ACV (0.2 ug/mL)	HSV-2+ACV (1.0 ug/mL)
0.2	12,627±367 (100 %)	7,081±868 (56 %)	4,521±94 (36 %)
0.5	16,564±201 (100 %)	12,231±811 (74 %)	6,744±91 (41 %)
1.0	17,360±619 (100 %)	11,529±1,351 (66 %)	7,403±372 (43 %)

Numbers in parenthesis indicate percentage of the average counts of ^{3}H-TdR incorporation in the HSV-2 infected cultures in the presence of acyclovir as compared to the parallel HSV-2 infected cultures in the absence of acyclovir

Table 4 In vitro inhibitory effect of acyclovir on DNA synthesis of HSV-2 infected cells at various multiplicities of infection.

MOI	Mean of count per minute ± SE								
	Doses of acyclovir (ug/mL)								
	0	0.05	0.1	0.2	0.5	1.0	2.0	3.0	4.0
2.5	6660±148 (100%)	5770±737 (86 %)	5623±217 (84 %)	4423±1034 (66 %)	5043±368 (75 %)	5307±258 (79 %)	5232±585 (78 %)	5139±84 (77 %)	4867±274 (73 %)
5	4369±95 (100%)	4204±287 (96 %)	3566±126 * (82 %)	3618±194 * (83 %)	3356±184 * (77 %)	3369±136 * (77 %)	3109±161 * (71 %)	2819±224 * (64 %)	2760±160 * (63 %)
10	3352±250 (100%)	3564±305 (106%)	3094±212 (92 %)	2357±107 * (70 %)	2565±107 * (76 %)	2410±105 * (72 %)	1918±48 * (57 %)	1895±79 * (56 %)	2039±147 * (60 %)
25	3872±185 (100%)	3356±405 (87 %)	3444±354 (89 %)	2598±100 * (67 %)	2452±86 * (63 %)	2120±29 * (55 %)	1677±46 * (43 %)	1646±39 * (43 %)	1538±31 * (40 %)

* $P < 0.05$ (Paired-t-test) compared with control.

Value in parenthesis indicates percentage of the average counts of ^{3}H-TdR incorporation in the HSV-2 infected cultures in the presence of acyclovir as compared to the parallel HSV-2 infected cultures in the absence of acyclovir.

Table 5 In vitro inhibitory effect of acyclovir on DNA synthesis of HSV-2 infected cells at different time-course of infection.

Hour Post infection (MOI=10)	Mean of count per minute ± SE								
	Doses of acyclovir (ug/mL)								
	0	0.05	0.1	0.2	0.5	1.0	2.0	3.0	4.0
1.5	3352±250 (100%)	3564±305 (106%)	3094±212 (92 %)	2357±107 * (70 %)	2565±107 * (76 %)	2410±105 * (72 %)	1918±48 * (57 %)	1895±79 * (56 %)	2039±147 * (60 %)
6	4597±177 (100%)	3310±103 * (72 %)	3043±164 * (66 %)	2716±151 * (59 %)	1949±108 * (42 %)	1736±40 * (38 %)	1321±80 * (29 %)	1331±48 * (29 %)	1162±13 * (25 %)
12	2845±114 (100%)	2620±94 (92 %)	2575±96 * (90 %)	2397±41 * (84 %)	1980±72 * (69 %)	1577±20 * (55 %)	1368±37 * (48 %)	1251±33 * (44 %)	1142±39 * (40 %)

* $P < 0.05$ (Paired t-test) compared with control.

Value in parenthesis indicates percentage of the average counts of ^{3}H-TdR incorporation in the HSV-2 infected cultures in the presence of acyclovir as compared to the parallel HSV-2 infected cultures in the absence of acyclovir.

Table 6 A comparison of the inhibitory effect of pure chemical acyclovir and intravenous acyclovir on the multiplication of the HSV-2 by plaque reduction assay at 6.0 h post infection.

Form of ACV	Virus yield : No. of PFU/mL								
	Concentrations of ACV (ug/mL)								
	0	0.05	0.1	0.2	0.5	1.0	2.0	3.0	4.0
Pure chemical	2.7×10^4	3×10^3	1×10^3	0	0	0	0	0	0
Intravenous	3.5×10^4	6×10^3	1×10^3	4×10^3	2×10^3	0	0	0	0

Table 7 A comparison of the inhibitory effect of pure chemical acyclovir and intravenous acyclovir on DNA synthesis of HSV-2 infected cells.

Form of ACV	Mean of count per minute ± SE								
	Concentrations of ACV (ug/mL)								
	0	0.05	0.1	0.2	0.5	1.0	2.0	3.0	4.0
Pure chemical	3590±166 (100%)	2988±132* (83 %)	2874±128* (80 %)	2558±146* (71 %)	2334±135* (65 %)	1958±144* (54 %)	1580±37* (44 %)	1195±90* (33 %)	1071±100* (29 %)
Intravenous	4597±177 (100%)	3310±164* (72 %)	3043±164* (66 %)	2716±151* (59 %)	1949±108* (42 %)	1736±40* (38 %)	1321±80* (29 %)	1331±48* (29 %)	1162±13* (25 %)

* $P < 0.05$ (paired t-test) compared with control

Value in Parenthesis indicated percentage of the average count per minute of ^{3}H-TdR incorporation in the HSV-2 infected cultures in the presence of acyclovir as compared to the parallel HSV-2 infected cultures in the absence of acyclovir.

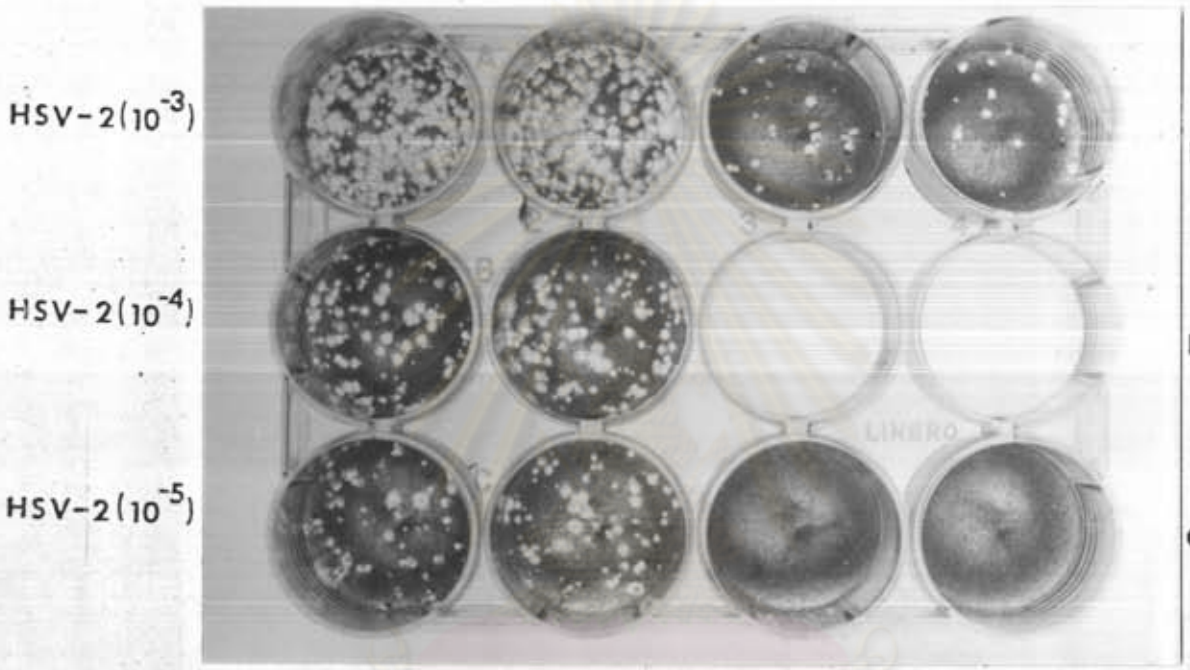

Fig 1 Titration of HSV-2. Monolayers of HeLa cells were infected with HSV-2 dilution of 10^{-3} to 10^{-6}, plaques were developed after 3 days of incubation at 37°C

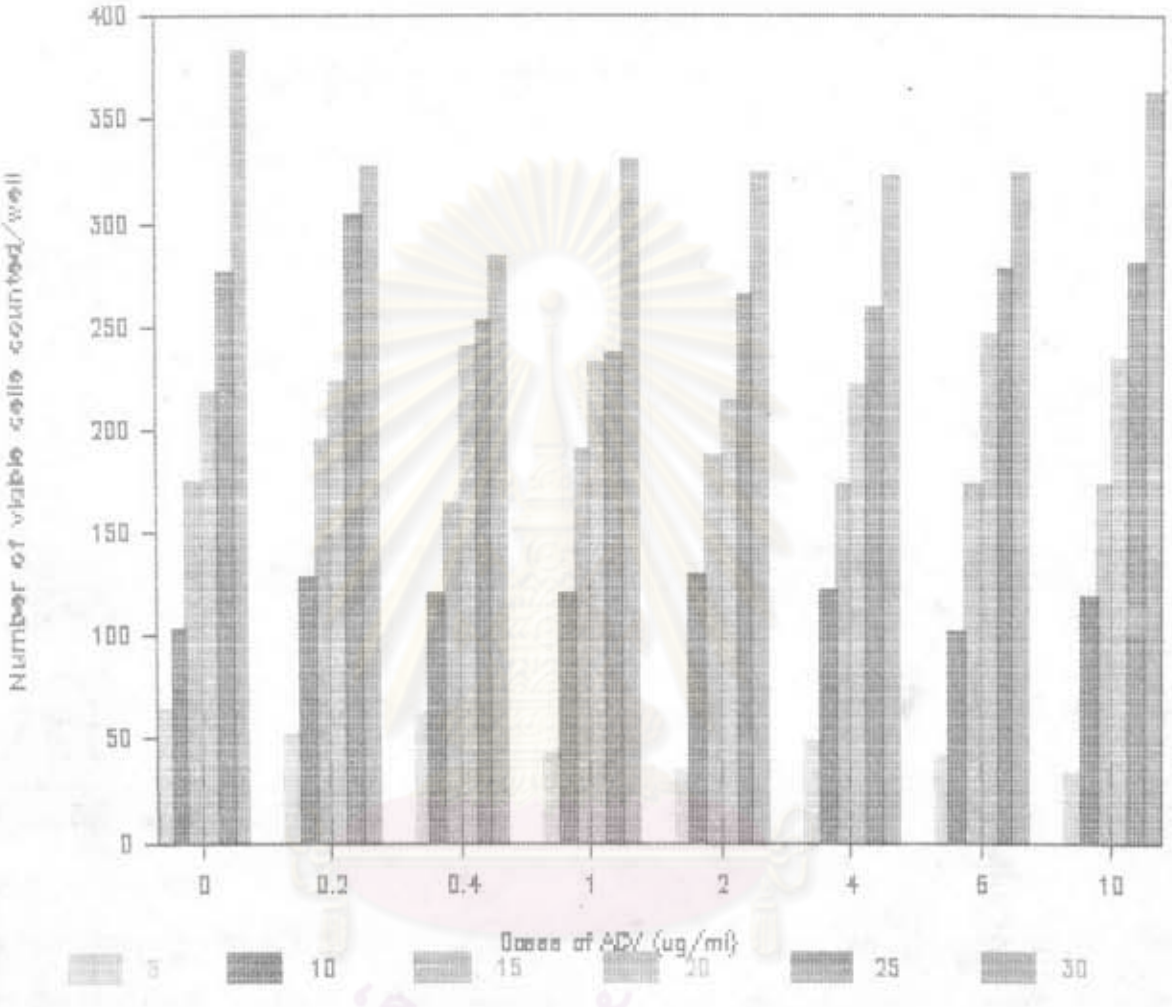

Fig 2 Toxic effects of acyclovir on HeLa cells.

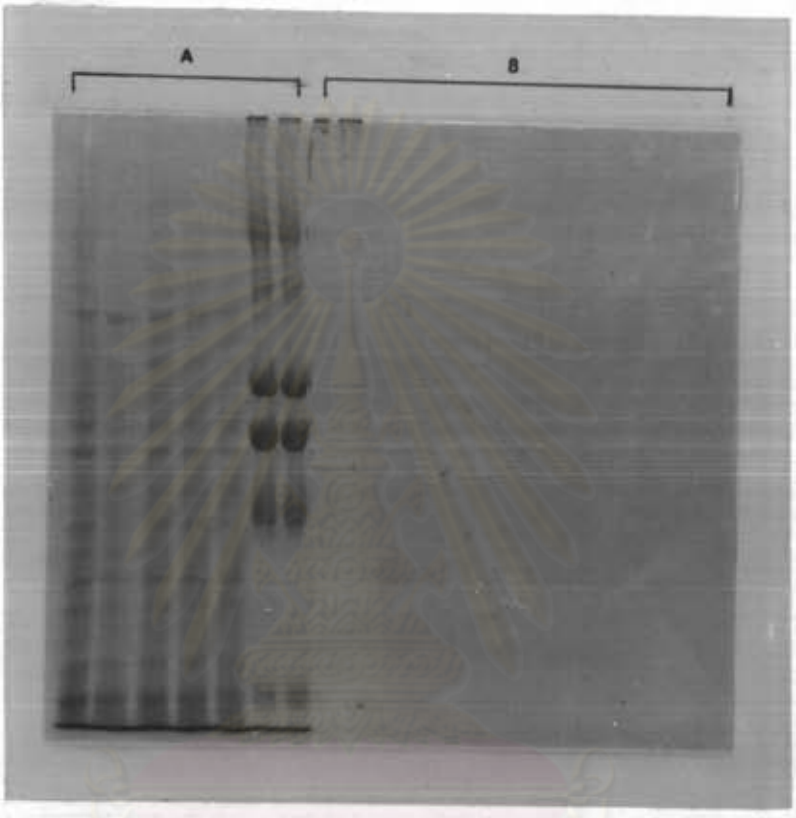

Fig 3 The SDS-PAGE was stained with 0.25 % Coommassie brilliant blue before (part A) and after (part B) an electrotransfer onto the nitrocellulose.

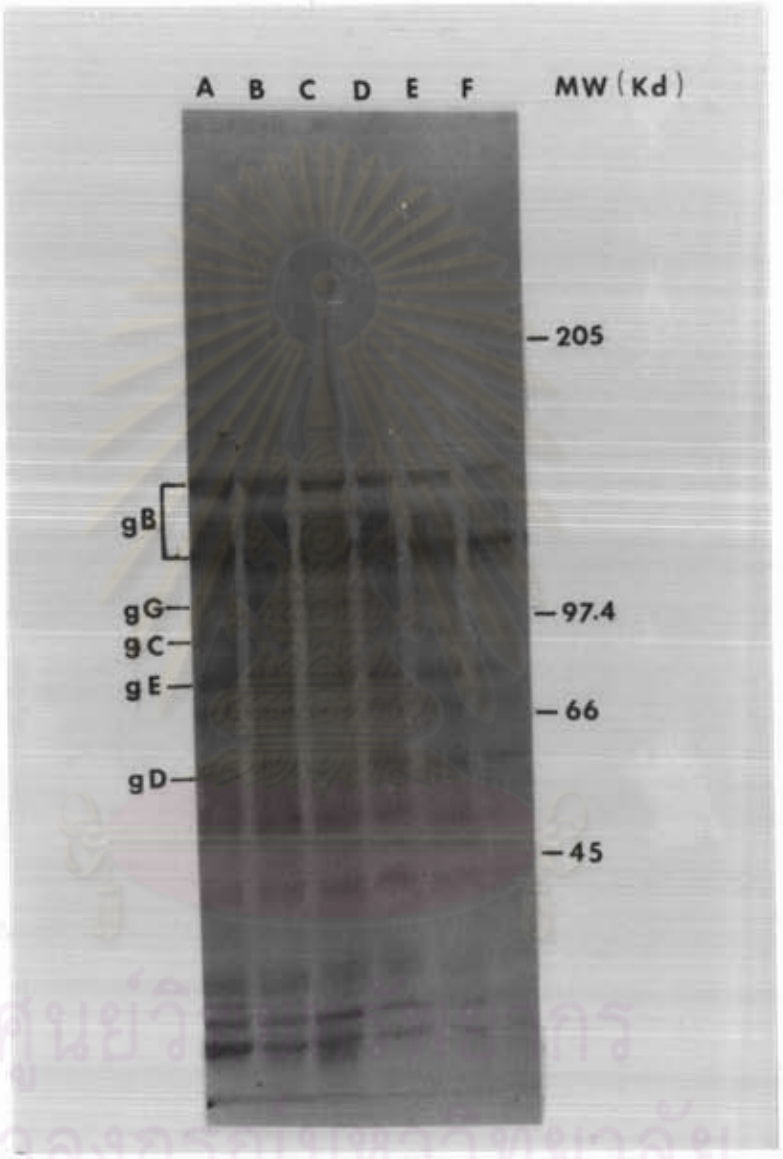

Fig 4 Inhibitory effect of different concentrations of acyclovir on the HSV-2 polypeptide synthesis in HeLa cells at 1.5 h post infection. HeLa cell monolayers infected with HSV-2 were either untreated (lane A) or treated with acyclovir at the concentrations of 0.2 ug/mL (lane B), 0.5 ug/mL (lane C), 1.0 ug/mL (lane D), 2.0 ug/mL (lane E) and 3.0 ug/mL (lane F). Following an incubation for 24 h, infected cell were electrophorosed and then analysed by Western blot assay. Numbers on the right represent the molecular weights in kilodaltons.

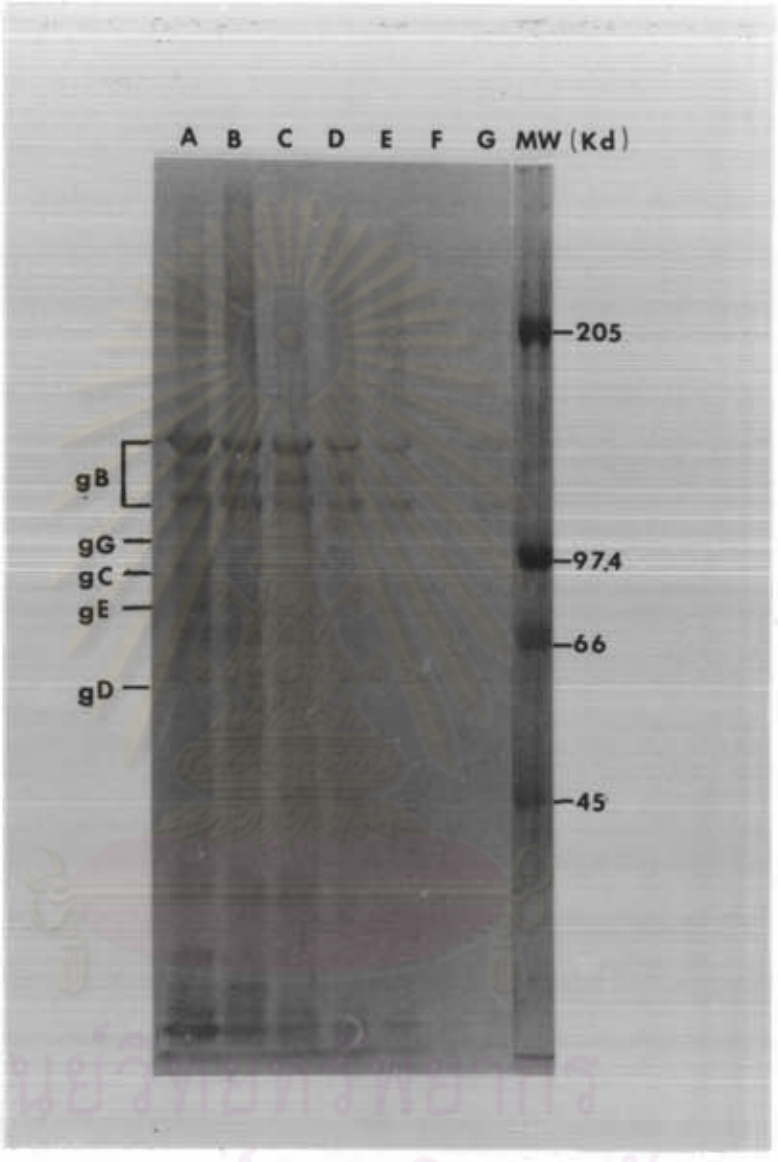

Fig 5 Inhibitory effect of different concentrations of acyclovir on the HSV-2 polypeptide synthesis in HeLa cells at 6.0 h post infection. The infected cells were treated with acyclovir at concentrations of 0 ug/mL (lane A), 0.2 ug/mL (lane B), 0.5 ug/mL (lane C), 1.0 ug/mL (lane D), 2.0 ug/mL (E), 3.0 ug/mL (lane F), and 4.0 ug/mL (lane G), respectively.

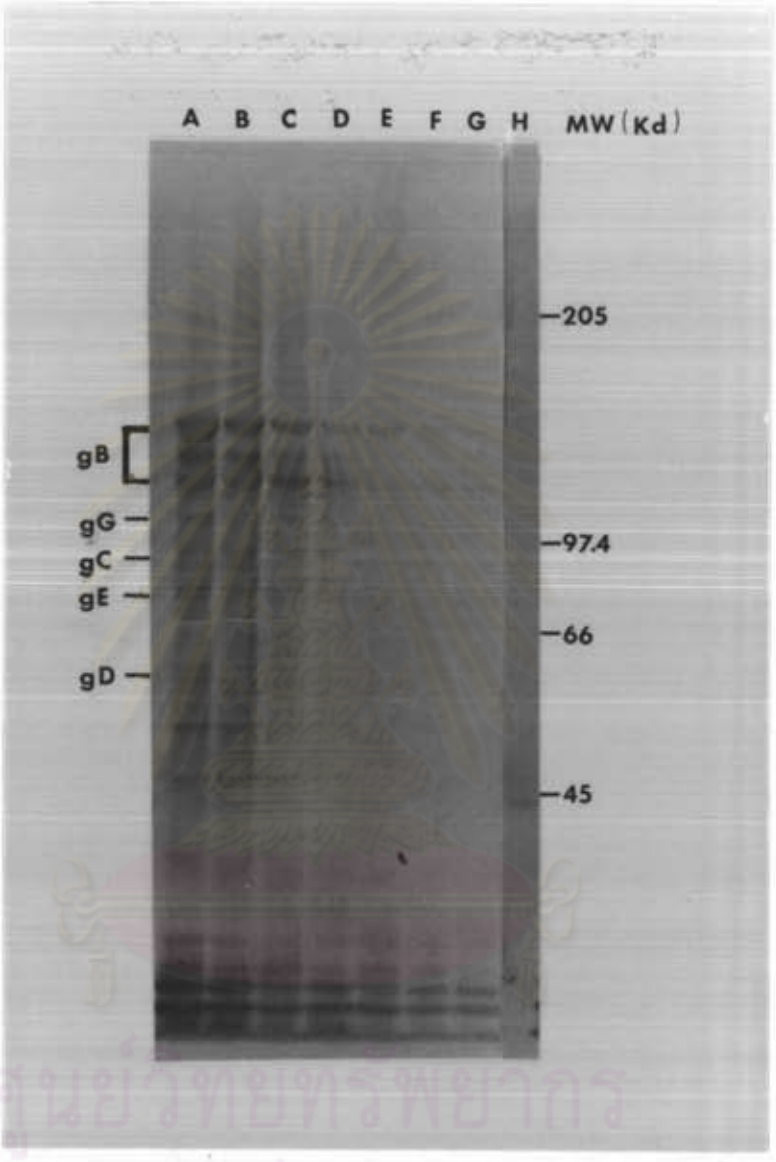

Fig 6 Inhibitory effect of different concentrations of acyclovir on the HSV-2 polypeptide synthesis in HeLa cells at 12.0 h post infection. The infected cells were treated with acyclovir at concentrations of 0 ug/mL (lane A), 0.2 ug/mL (lane B), 0.5 ug/mL (lane C), 1.0 ug/mL (lane D), 2.0 ug/mL (lane E), 3.0 ug/mL (lane F) and 4.0 ug/mL (lane G), respectively. Lane H was mock infected culture.

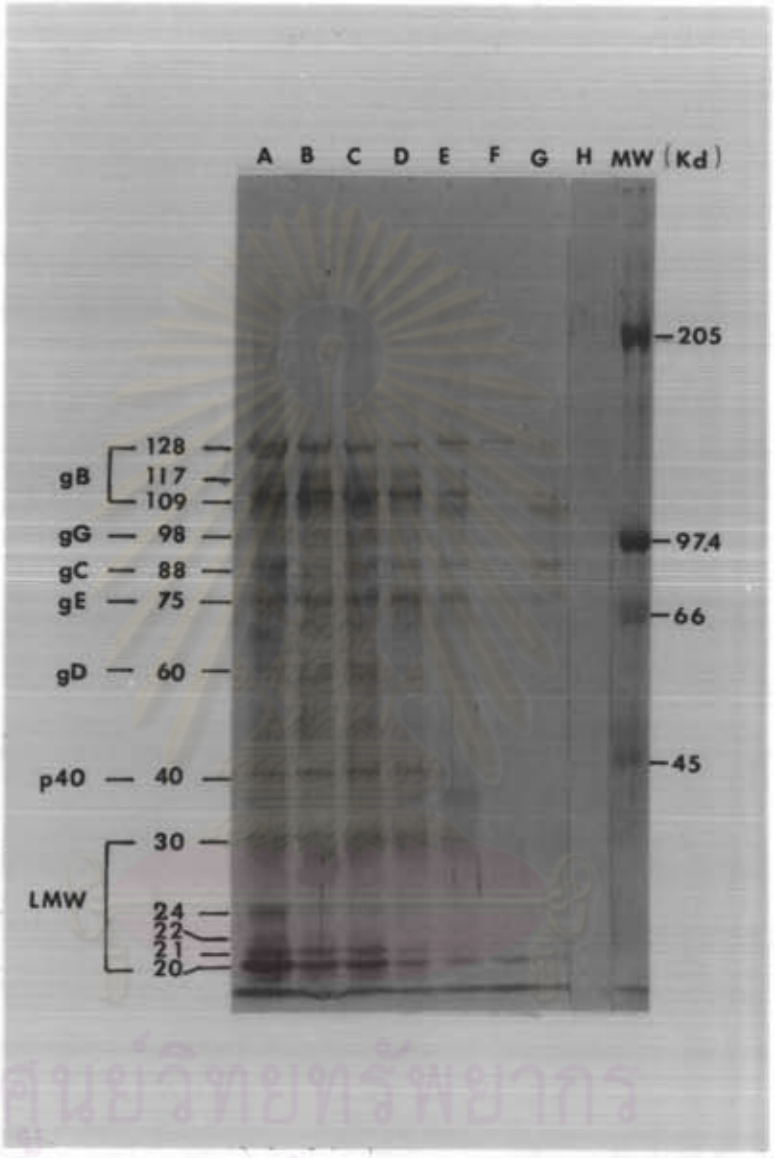

Fig 7 Inhibitory analysis of inhibitory effect of pure-chemical acyclovir on HSV-2 polypeptide synthesis in HeLa cells at 6.0 h post infection. HSV-2 infected culture were treated with acyclovir at concentrations of 0, 0.2, 0.5, 1.0, 2.0, 3.0 and 4.0 ug/mL in Lane A,B,C,D,E,F and G respectively. Lane H was mock infected culture.