BIKEN'S JOURNAL 3, 57-76 (1960)

Studies on the Inhibitory Activity of 5, 6-Dichloro-I-β-D-ribofuranosylbenzimidazole (DRB), Ribonuclease and Proflavine on the One-Step Growth Cycle of Mouse Pox Virus (Ectromelia Virus) in L Cell Tissue Culture*

NOBUKO IKEGAMI, SHIRO KATO AND JUNTARO KAMAHORA

Department of Pathology, the Research Institute for Microbial Diseases, Osaka University, Osaka

(Received for publication, March 19, 1960)

SUMMARY

The effects of three metabolic inhibitors, that is, DRB, RNase and proflavine, upon the one-step growth cycle of mouse pox virus (ectromelia virus) in L cell tissue culture were studied. Infectivity of progeny virus (plaque forming unit) and complement fixing antigen as well as inclusion body formation were studied in the presence of these compounds. All three compounds were found to have a considerable inhibitory effect upon the production of infective virus at concentrations which allow uninhibited multiplication of the cells. The mechanism of the action of these compounds were analyzed by changing the time and duration of administration of the compounds in the one step cycle of virus growth. The analysis has revealed that the modes of action of DRB and RNase are very similar, while that of proflavine is very different from that of the other two compounds. The former two compounds were only effective when the host cells were exposed to them before virus inoculation, indicating that a metabolic disturbance in RNA caused in the host cells is enough to suppress viral DNA synthesis. These compounds have no significant effects upon the further virus maturation mechanism. On the contrary, pretreatment of the host cells by proflavine does not have any noticeable effect upon virus multiplication, while the administration of the compound at any time after virus inoculation caused apparent suppression of the maturation process of the progeny virus. Morphologically, proflavine has little effect upon the initiation of "B" type inclusion formation. However, most of the "B" type inclusions formed in the presence of proflavine were small and compact, and further development to the large granular network-like structure was greatly inhibited. Proflavine treated "B" type inclusions gave the same Feulgen positive reaction and positive fluorescent antibody reaction as those of untreated inclusions. This test indicates the site of accumulation of viral components. The removal of proflavine from the system reversibly allowed the further development of "B" type inclusions and the maturation of progeny virus.

INTRODUCTION

In this laboratory, for several years, studies on the mechanism of multiplication of various pox group viruses propagated *in vivo* and *in vitro* have been carried out especially from the morphological stand point (Kato 1955; Kamahora *et al.*, 1955; Hagiwara and Kamahora, 1956; Furusawa *et al.*, 1958; Kato and

* This report was presented at the Annual Meeting of the Society of Japanese Virologists, in Tokyo, on October 16, 1959.

Cutting, 1959; Kato *et al.*, 1959). An inclusion named the "B" type has been found which is morphologically common to infections of all pox group viruses examined. The "B" type inclusions were Feulgen positive and also proved to be the site of viral antigen by Coon's fluorescent antibody technique (Takahashi *et al.*, 1959; Kameyama *et al.*, 1959).

Recently autoradiographic study on the cells infected with pox group viruses with tritium-labeled thymidine has revealed that most of the sites of aggregation of silver granules in the cytoplasm corresponded to "B" type inclusions (Kato *et al.*, 1960).

Therefore, it is reasonable to regard the "B"type inclusions as the site of virus production and to assume these viruses to be DNA viruses.

The "B" type inclusion of ectromelia virus was first described by Kato, Hagiwara and Kamahora (1955). Further histochemical and histoimmunological studies have revealed that the "B" type inclusions of this virus possess the same characteristics as those of other viruses. Thus the "B" type inclusions provided us with a new method for estimation of the infected cells, and their morphological observations allowed us to estimate the stage or degree of infection of the cells (Kamahora *et al.* 1959). Besides "B" type inclusions, ectromelia virus produces secondary inclusions named the "A" type (Marchal bodies) which do not seem to play any essential role in virus multiplication. The appearance of the "A"type inclusion is labile, depending upon the condition of the host cell and its environment.

The scheme of morphological development of the two kinds of inclusions is shown in Fig. 1.

In a study on the relationship between cell mitosis and inclusion formation, Kamahora *et al.* (1957) found that the mitosis of cells infected with ectromelia virus was suppressed by some effect of virus infection.

It is of interest to analyze the correlation between virus growth and cellular metabolism. In the present report, three metabolic inhibitors, that is, 5, 6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), ribonuclease (RNase) and proflavine were used in the ectromelia virus L cell system in tissue culture.

This is the first trial in which virus inclusions were used as specific indicators of virus infection of a single cell. The one-step growth cycle of the DNA animal virus is used for the study of the activity of the compounds.

A short communication of these studies has already been reported (Ikegami *et al.*, 1959).

MATERIALS AND METHODS

1. *Virus*

Virus suspensions used throughout the present work were the supernatants after centrifugation of a 30 per cent liver emulsion of the moribund mice, infected with the Hampstead strain of ectromelia virus. The infectivity of this material was about 10^8 plaque forming units per ml.

2. *Cells*

Earle's L cells (derived from mouse fibroblast cells) were maintained by culture on glass.

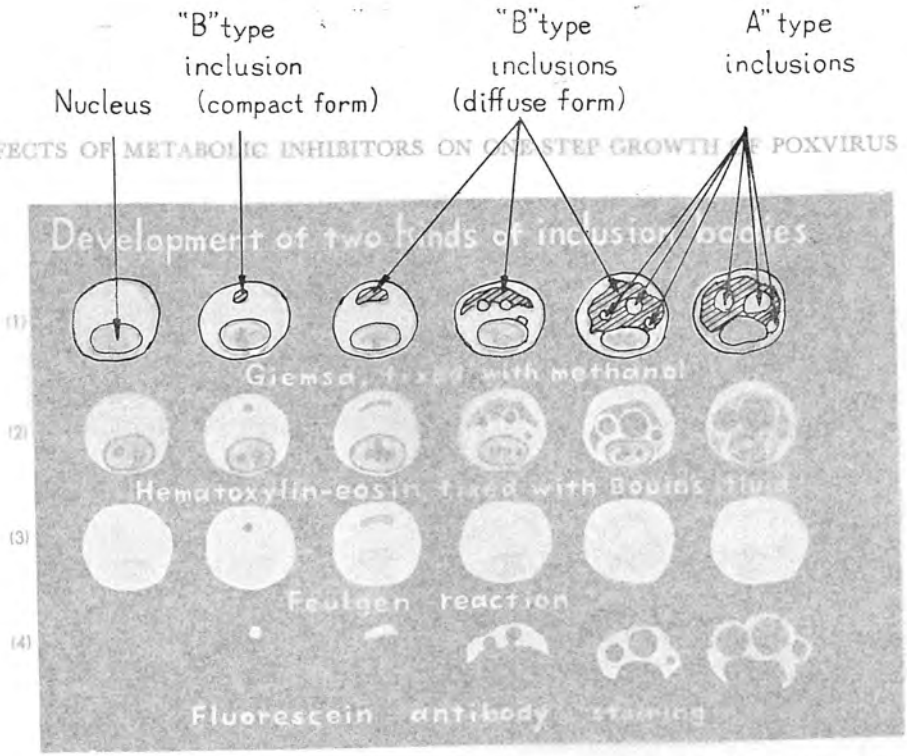


Fig. 1. Scheme of development of two kinds of inclusion bodies and the appearance of the "A" and "B" type inclusions in various cytological procedures.

(1) Giemsa staining after methanol fixation : "B" stains reddish purple. "A" stains faint reddish granular on a pale blue back ground.

(2) H-E staining after Bouin's fluid fixation : "B" stains hematoxylinophilic with combined eosin tinge, surrounded by a halo. "A" stains bright red, surrounded by a halo.

(3) Feulgen reaction : Especially nucleus and "B" show positive reaction.

(4) Fluorescent antibody reaction : "B" shows definite fluorescence.

3. *Culture medium*

The growth medium was composed of 95 parts La-Ye Hanks' solution containing 0.5 per cent lactalbumin hydrolysate, 0.1 per cent yeast extract and 5 parts of inactivated bovine serum and antibiotics (100 units of penicillin and 100 μg of streptomycin per ml).

4. *Metabolic inhibitors*

1) *DRB*

The benzimidazole derivative, 5,6-dichloro-I-β-D-ribofuranosylbenzimidazole (DRB) was kindly provided by Dr. Folkers of Merck and Company Inc. through the courtesy of Prof. Takagi of Kanazawa University.

2) *Ribonuclease*

This was a commercial product. (Worthington Biochemical Corp.).

3) *Proflavine*

Proflavine (3,6-diaminoacridine hydrochloride) was kindly supplied by Prof. Ouring of Stanford University. This was dissolved in Hanks' solution and stored in the dark at 4°C before use.

5. *Antisera*

1) *Hyperimmune ascites fluid of mice*

Following the method of Furusawa *et al.* previously described (1956), mice

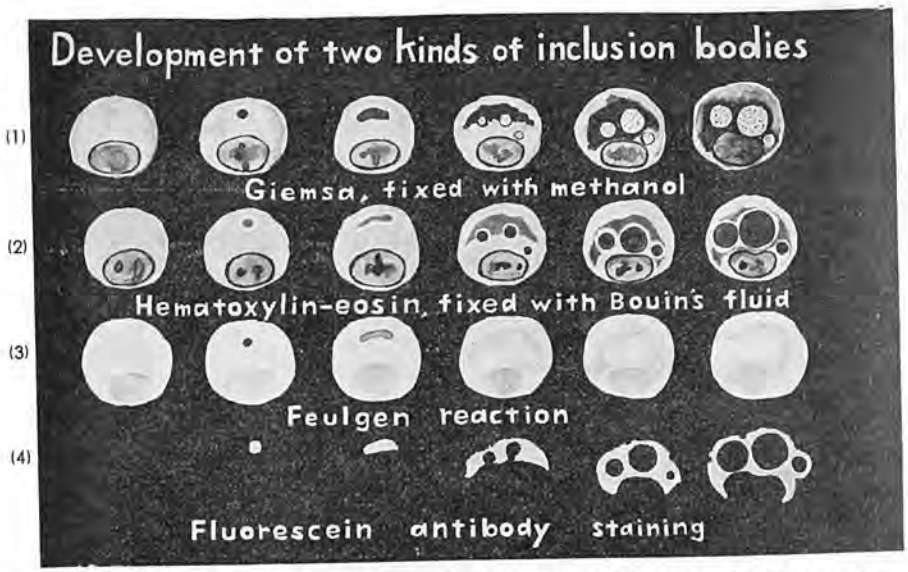


Fig. 1. Scheme of development of two kinds of inclusion bodies and the appearance ot the "A" and "B" type inclusions in various cytological procedures.
(1) Giemsa staining after methanol fixation : "B" stains reddish purple. "A" stains faint reddish granular on a pale blue back ground.
(2) H-E staining after Bouin's fluid fixation : "B" stains hematoxylinophilic with combined eosin tinge, surrounded by a halo. "A" stains bright red, surrounded by a halo.
(3) Feulgen reaction : Especially nucleus and "B" show positive reaction.
(4) Fluorescent antibody reaction : "B" shows definite fluorescence.

3. *Culture medium*

The growth medium was composed of 95 parts La-Ye Hanks' solusion containing 0.5 per cent lactoalbumin hydrolysate, 0.1 per cent yeast extract and 5 parts of inactivated bovine serum and antibiotics (100 units of penicillin and 100 μg of streptomycin per ml).

4. *Metabolic inhibitors*

1) *DRB*

The benzimidazole derivative, 5,6-dichloro-I-β-D-ribofuranosylbenzimidazole (DRB) was kindly provided by Dr. Folkers of Merck and Company Inc. through the courtesy of Prof. Takagi of Kanazawa University.

2) *Ribonuclease*

This was a commercial product. (Worthington Biochemical Corp.).

3) *Proflavine*

Proflavine (3,6-diaminoacridine hydrochloride) was kindly supplied by Prof. Cutting of Stanford University. This was dissolved in Hanks' solution and stored in the dark at 4°C before use.

5. *Antisera*

1) *Hyperimmune ascites fluid of mice*

Following the method of Furusawa *et al.* previously described (1956), mice

were given two or three intraperitoneal injections of virus materials inactivated by ultraviolet irradiation at weekly intervals. This injection was followed a week later by one injection of active virus by the same route.

Survivors were inoculated intraperitoneally with Ehrlich ascites tumor cells 10 days after the last injection. Ascites fluid was harvested from inoculated mice, and a clear fluid was obtained by centrifugation. This supernatant fluid was heat inactivated at 56°C for 30 minutes and was used as the antibody in the determination of complement fixing antigen. Complement fixing titers of the immune ascites fluid were obtained up to 120 times dilution.

2) *Anti-virus hamster serum*

Hamsters were immunized with a 10 per cent emulsion of chorioallantoic membrane infected with ectromelia virus. Immunization procedures were carried out by injection with active virus three times, at weekly intervals.

3) *Anti-hamster globulin rabbit serum*

Rabbits were immunized with globulin of normal hamsters. After two injections with Freunds' adjuvant, rabbits were bled by cardiac puncture. The globulin fraction of immune rabbit serum was coupled with fluorescence isothiocyanate according to Marshall's method (1958).

6. *Biological test.*

Infected cell suspensions of at least two or three tissue culture tubes were pooled and subjected to freezing and thawing for more than ten times. The centrifuged extracts were used for the determination of complement fixing antigen and the titers of infectious virus was measured by the plaque assay technique.

1) *Complement fixation test*

A modification of Ogata's syphilis reaction was employed in this test. 10 units of antibody in 0.25 ml and 2 units of guinea pig complement in 0.25 ml were added to tubes each containing 0.25 ml of serial dilutions (two fold) of antigen. The test tubes were stored at 4°C overnight, and then a hemolytic system, composed of 3 per cent cow red cells sensitized by 7 units of anti-cow red cell rabbit serum was added.

2) *Virus assay*

The plaque test for infectious ectromelia virus has been described in detail previously (Nii, 1959). 5 ml aliquots of L cell suspension containing about 2.5×10^5 cells per ml were implanted into a 50 ml bottle. Continuous cell sheets were formed after one to two days culture. After removal of the culture medium, the L cell monolayers were washed twice with 2 ml of Hanks' solution and then 0.5 ml of serial dilutions (ten fold) of sample and 1 ml of Hanks' solution were added to the cell layers. Afer three hours exposure to virus at 37°C, the extracellular fluids were removed from the cell layers, and the cell sheets were washed three times and covered with a melted agar overlay mixture. The bottles were incubated at 37°C. Plaques were counted on the tenth day. Results were expressed as plaque forming units per ml (PFU/ml).

A simpler method was also employed for virus assay. After the virus adsorption period, the cell sheets were cultivated with 5 ml of La-Ye Hanks' solution containing 3 per cent bovine serum in place of the agar overlay. Primary foci forming on the cell sheets were counted on the fifth day. The results obtained by the two methods were in good accordance for the assay of ectromelia virus.

3) *Morphological observations*

A. *Observations on inclusions*

Infected cells in the remaining two test tubes, other than those employed for biological assay, were used to make a smear preparation. This preparation was fixed with methanol

and stained with Giemsa solution, and was used to count the number of inclusion bearing cells.

B. *Histochemical and histoimmunological observations on the inclusions*

Coverslip cultures were prepared. The Feulgen reaction on the cells was used for histochemical study, and the indirect fluorescent antibody staining method of Weller and Coons (1954) for histoimmunological study.

7. *Experimental procedure*

1) *Cell culture and addition of metabolic inhibitors*

One ml of cell suspension, containing about 2.5×10^5 cells per ml, was implanted per tube. The test tubes were incubated in the stationary state at 37°C for several days. 0.1 ml of Hanks' solution containing various concentrations of each metabolic inhibitor was added to each tube in the experimental group. Control tube received 0.1 ml Hanks' solution only.

The effect of these metabolic inhibitors on the cell proliferation was determined by cell counting at daily intervals.

2) *Virus inoculation*

The culture medium was removed from the test tubes 48 hours after cell implantation and 0.5 ml of virus suspension having $10^{7.5}$ PFU/ml was inoculated into each tube. Two hours incubation at 37°C was allowed for virus adsorption, and then the medium containing residual viruses was decanted, and the cells were washed three time with Hanks' solution to remove most of the unadsorbed virus. Samples were taken fifteen or sixteen hours after virus inoculation for biological assay.

8. *Classification of experimental groups*

To analyze the mode of inhibition of the compounds on virus reproduction, the compounds were added at various time intervals after cell implantation as summarized in Fig. 2.

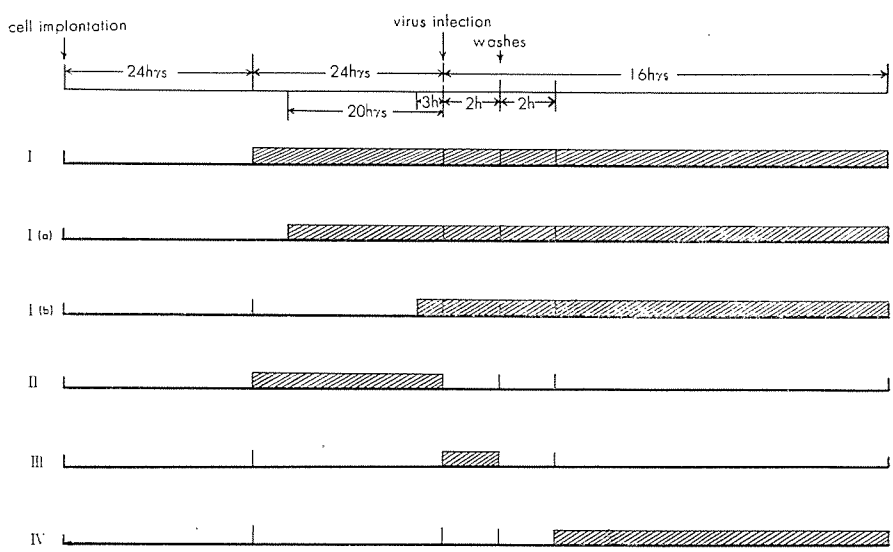


Fig. 2 Classification of Experimental Groups Depending on the Time Period of Addition of the Compounds

Untreated infected cultures served as controls. In group I, the compound was added to the culture tube 24 hours before virus infection, and the cells were exposed to the compound throughout the experimental period. Groups I(a) and I(b) were adopted only in the experiments with RNase. In group I(a), RNase was added 20 hours before virus inoculation and in group I(b) 3 hours before.

The cells in both groups were exposed to RNase until samples were taken for biological assay. In group II, the cells were brought into contact with the compound only in the period before virus inoculation, to see if there was an influence of the compound on the cellular metabolism which might affect virus multiplication. In group III, the compound was added to the culture tube only during the period of virus adsorption. Group III probably shows the direct action of the compound on the infectious virus particles and on their adsorption and invasion into the cells. In group IV, the cells were exposed to the compound from 4 to 16 hours after virus inoculation to see the effect of the compound in the later stages when the viral components were being synthesized in the cell.

RESULTS

1. *Effect of DRB on cell proliferation and on virus multiplication*

The effect of DRB on cell proliferation is shown in Fig. 3.

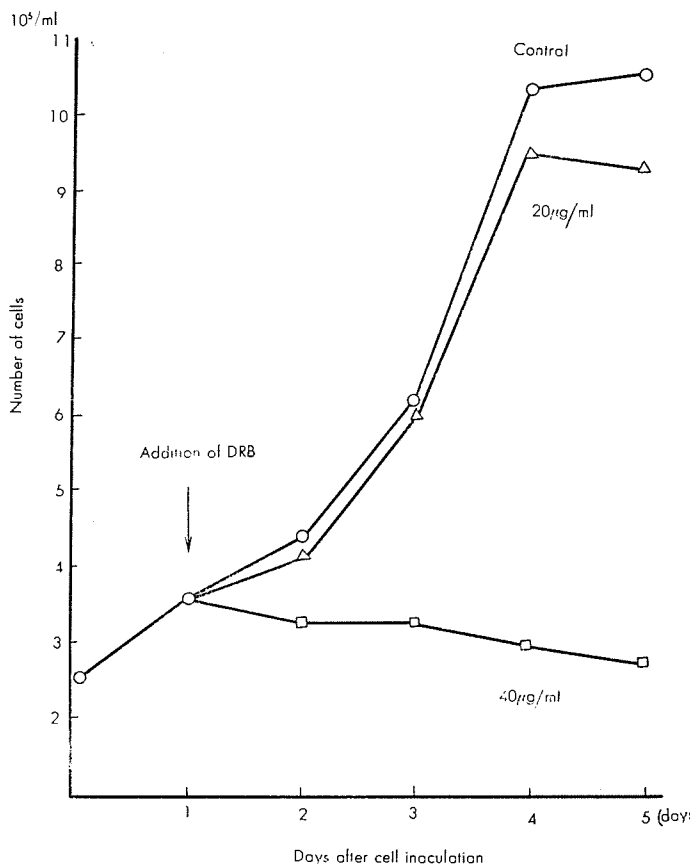


Fig. 3 Effect of Dose of DRB on Proliferation of L Cells

DRB at the concentration of 20 μg per ml did not affect cell growth for a certain period. On the other hand, although, at a concentration of 40 μg per ml, cell proliferation was suppressed from the time of its addition, no severe degenerative changes of the cells were noticed microscopically.

These two dosages of DRB, which showed different effects on cell proliferation, were employed to analyze the action of DRB on virus multiplication. The results are summarized in Table 1.

Table 1. Comparison of Percentage of Inclusion Bearing Cells, Complement Fixing Antigen, and Infectivity in the Experiments with DRB

Dose μg/ml	Experimental group	Percentage of inclusion cells (%)	C.F.A.	Infectivity (PFU/ml)
0	control	91	64	1.7×10^5
20	I	36	32	1.4×10^4
	II	59	32	1.7×10^4
	III	91	64	1.7×10^5
	IV	89	64	1.6×10^5
0	control	84	64	1.5×10^5
40	I	20	16	2.4×10^3
	II	38	32	1.5×10^4
	III	78	64	1.5×10^5
	IV	50	32	1.6×10^4

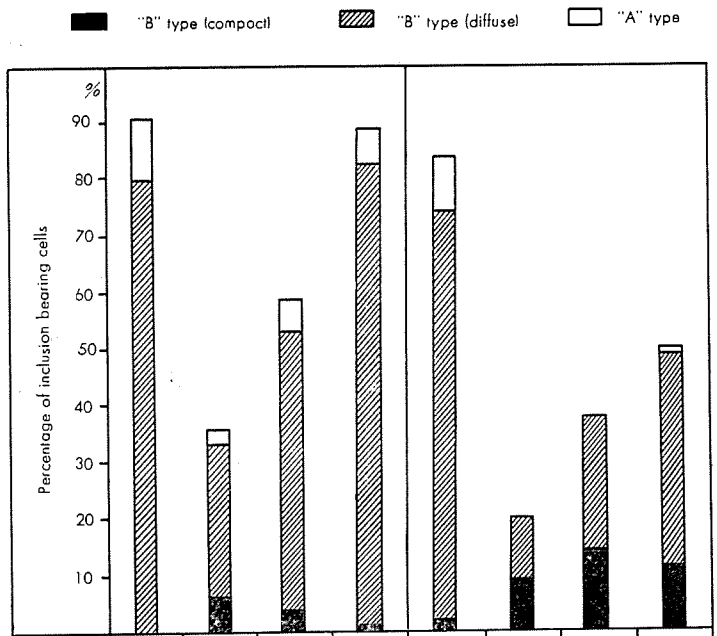
note : Samples were taken sixteen hours after virus inoculation for biological assays.

Neither dosage of DRB had any effect on infectious virus particles themselves or on their adsorption and invasion into cells in experimental group III.

In the cultures which were pretreated with 20 μg per ml (see experimental groups I and II), the amount of complement fixing antigen and the number of inclusion bearing cells were reduced to about half or one third of those in the untreated cultures. In spite of the remarkable decrease in the number of inclusion bearing cells, the inclusions which appeared in the treated cell regularly showed normal developmental features, with a granular and network-like structure just like the inclusions in the untreated cells (see experimental groups I and II in Fig. 4).

The yield of new infectious virus also decreased to one tenth of the control value.

The presence of DRB (at the concentration of 20μg) after virus adsorption had no significant effect on the formation of the three viral components examined or on their development (see experimental group IV in Table I and Fig. 4).



Inclusion %	91	36	59	89	84	20	38	50
Exp. group	Control	I	II	IV	Control	I	II	IV
Dose (μg/ml)	0	20			0	40		

Fig. 4 Effects of DRB on Formation of Inclusions and Their Development

From these results, it seems that some disturbance of cellullar RNA metabolism caused before virus infection may be responsible for the depressed synthesis of virus constituents, containing DNA and various antigenic proteins.

Pretreatment with 40 μg per ml of DRB resulted in more inhibition of virus reproduction in the one-step growth cycle than that with 20 μg. Moreover, DRB suppressed virus multiplication when 40 μg (which was toxic to cell proliferation) was administered after virus infection.

2. *Effects of RNase on cell proliferation and on virus multiplication*

Experiments were carried out to ascertain whether a significant relationship existed between cellular RNA metabolism and the reproduction of DNA containing virus.

It is shown in Fig. 5 that cell proliferation is hardly affected by the presence of 1 mg or 2 mg per ml of RNase.

As shown in Table 2, pretreatment of the cells with these dosages resulted in a remarkable reduction in the formation of the three viral components examined.

The "B" type inclusions showing diffuse network-like structure decreased in

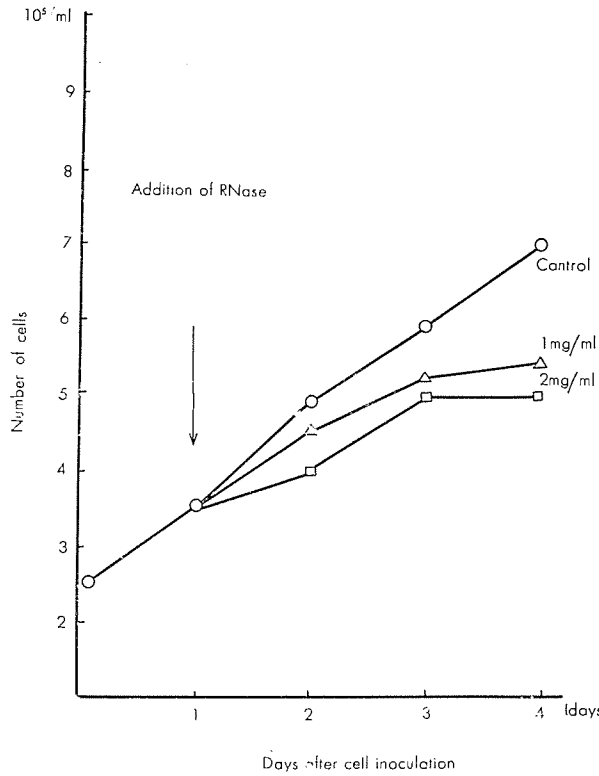


Fig. 5 Effect of Dose of RNase on Proliferation of L Cells

Table 2. Comparison of Percentage of Inclusion Bearing Cells, Complement Fixing Antigen, and Infectivity in the Experiments with RNase

Dose mg/ml	Experimental group	Percentage of inclusion cells (%)	C.F.A.	Infectivity (PFU/ml)
0	control	79	64	2.3×10^4
1	I (a)	20	16	1.4×10^3
	I (b)	22	16	1.5×10^3
	II	28	32	2.3×10^3
	III	74	64	2.0×10^4
	IV	72	64	2.0×10^4

note : Samples were taken fifteen hours after virus inoculation for the biological assay.

number, and the "A"type inclusions were never found in pretreated cells (see experimental groups I(a) and (b) and II in Fig. 6).

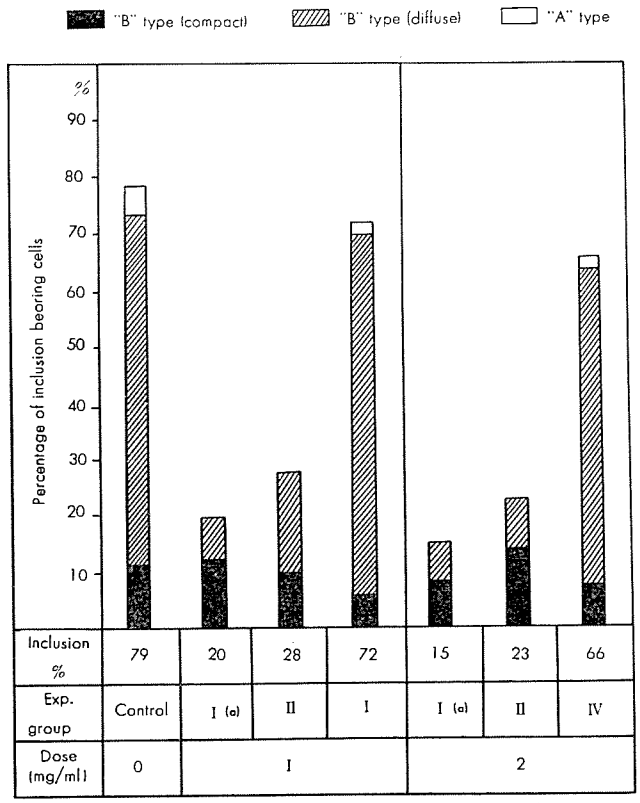


Fig. 6 Effects of RNase on Formatinn of Inclusions and Their Development

As with 20 μg of DRB, the presence of RNase after virus adsorption had little effect on the multiplication of the virus.

The effectiveness of DRB and RNase on virus reproduction was found to be very similar. However, the latter seemed to have a more rapid inhibitory effect because three hours pretreatment was enough to produce the same effect (experimental group I (b) in Table 2).

3. *Effects of proflavine on cell proliferation and virus multiplication*

The doses of proflavine which are and are not effective in inhibiting cell proliferation are shown in Fig. 7. At a concentration of 0.5 μg per ml, cell growth was not affected for at least three days, within which time its action on the one cycle of virus multiplication had ceased.

A concentration of more than 2.5 μg per ml of proflavine inhibited cell growth, and a granular cellular fluorescence produced by proflavine was detectable in the cytoplasm under the fluorescence microscopy after addition of a concentration of more than 5 μg per ml of proflavine.

To study the action of proflavine on virus multiplication, the experiments

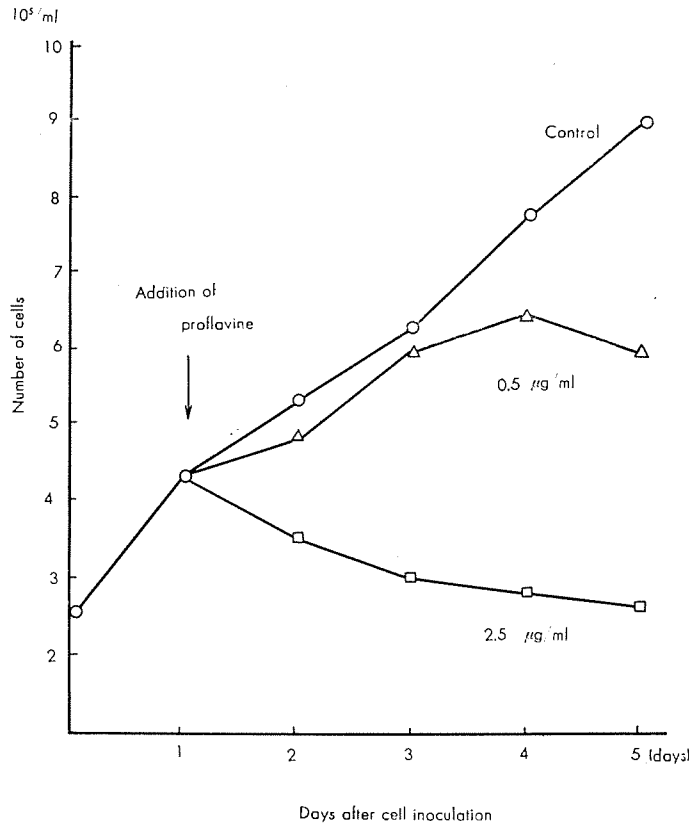


Fig. 7 Effect of Dose of Proflavine on Proliferation of L Cells

described above were carried out at both concentrations of proflavine. The results of these experiments are summarized in Table 3.

At the concentration of proflavine employed, there was no inhibition either of free virus particles or of their adsorption and invasion into the cells (experimental group III).

In exteriments with 0.5 μg proflavine per ml, the results differed from those with DRB and RNase in that the yield of new infectious virus was suppressed by both pre- and post-treatment with proflavine (experimental groups I, II and IV in Table 3), and the amount of complement fixing antigen and the number of inclusion bearing cells were unchanged as compared with the control.

Furthermore, it is of interest to note that "B" type inclusions of compact form were still observed in considerable numbers at a late stage of one cycle of virus multiplication. These inclusions are usually found at an early stage of infection in untreated cells (shown in Fig. 8)

When a concentration of 2.5 μg per ml proflavine was added to the cultures after virus adsorption (experimental group IV), this compound produced a more

Table 3. Comparison of Percentage of Inclusion Bearing Cells, Complement Fixing Antigen, and Infectivity in the Experiments with Proflavine

Dose μg/ml	Experimental group	Percentage of inclusion cells (%)	C.F.A.	Infectivity (PFU/ml)
0	control	91	64	1.5×10^5
0.5	I	79	64	2.4×10^3
	II	83	64	4.2×10^4
	III	91	64	1.5×10^5
	IV	79	64	1.5×10^4
2.5	I	36	32	1.0×10^3
	II	80	64	2.0×10^4
	III	90	64	1.5×10^5
	IV	65	64	2.0×10^3

note : Samples were taken sixteen hours after virus inoculation for the biological assay

pronounced effect on the production of infectious virus particles than when added before infection.

However, the formation of inclusions was reduced slightly, as compared with the decrease in yield of infectious viruses. In the culture both pre- and post-treated with 2.5 μg of proflavine (experimental group I in Table 3 and Fig. 8), there was some inhibition of formation of complement fixing antigen and of inclusions, and all "B" type inclusions were small and compact. These inclusions showed no diffuse network-like structure, which was usually seen at a late stage of infection in untreated cells.

To study the suppressive action of proflavine more exactly, it was added to the culture 4 hours or 6 hours after infection.

Samples were taken at various intervals, and the yield of infectious virus at each time estimated. The results are given in Fig. 9

There was a small increase in the amount of infectious virus within about 4 hours after addition of the compound, but a further rise in the infective titer could not be obtained thereafter.

These results show that the addition of proflavine at any stage after infection appears to prevent the further production of the infective virus.

4. *The cytochemical and cytoimmunological observation of inclusions in proflavine treated cells*

As mentioned previously, most of the "B" type inclusions observed in proflavine treated cells were compact and small even at a late stage of one cycle of virus growth (Fig. 14), and "A" type inclusions (Marchal bodies) could rarely be seen in the presence of 2.5 μg of proflavine by sixteen hours.

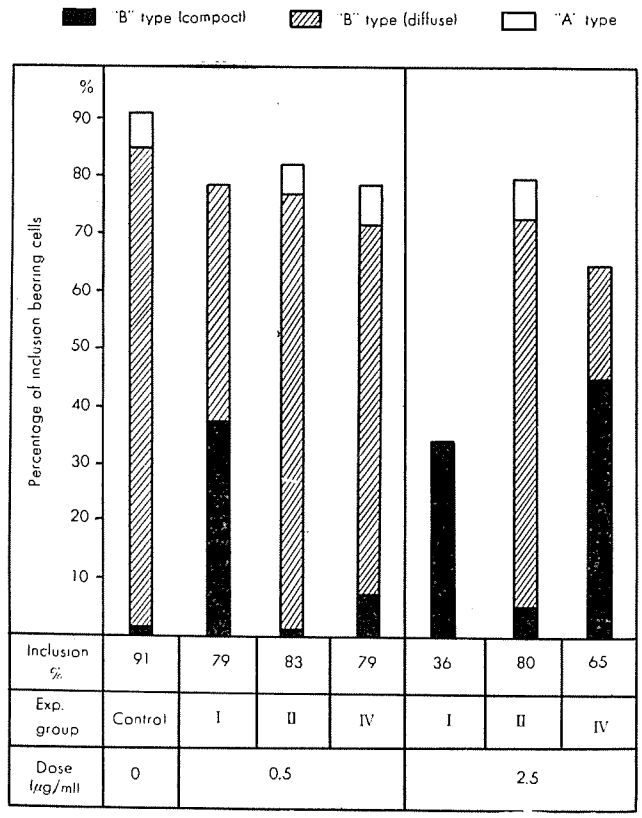


Fig. 8 Effects of Proflavine on Formation of Inclusions and Their Development

To see whether proflavine treated "B" type inclusions represented the main site of virus multiplication in the cells, like those in the untreated infected cells, cytochemical and cytoimmunological observations were made on these inclusions.

All proflavine treated "B" type inclusions gave the same degree of Feulgen positive reaction (Fig. 15) and positive fluorescent antibody reaction as those of untreated cells. Therefore, the presence of proflavine is unlikely to affect the initial formation of viral constituents, but may disturb their development to infectious virus particles.

5. *Reversal of inhibition of virus multiplication by removal of proflavine*

Virus multiplication in treated cells may recover from the depressed state after removal of proflavine, because pretreated cells did not undergo so pronounced an effect on the production of infectious virus as post-treated cells.

To investigate this possibility, the following experiments were carried out. Treated cells were cultivated in a mediun containing proflavine (at a concentration of 2.5 μg per ml) for the first 8 hours after infection. The cells were then washed twice with Hanks' solution and covered with a medium free of proflavine for the

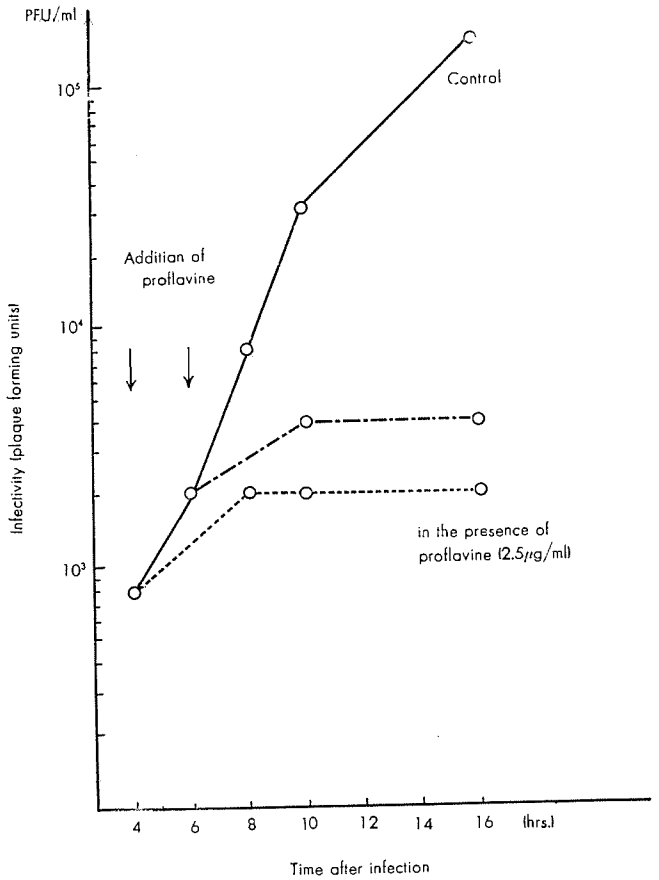


Fig. 9 Effect of Proflavine Added after Infection on the Yield of Infectious Virus Particles

following 16 hours. Two kinds of control cultures containing infected cells were prepared simultaneously: throughout the incubation period (the incubation period in these experiments was 24 hours), one was cultivated in a medium containing proflavine, the other in its absence.

Samples were taken at appropriate intervals from the cultures in each group, and the number of inclusion bearing cells and the infectivity measured. The results are presented in Figs. 10 and 11.

It was found that the "B" type inclusions in 8 hour proflavine treated cells lost their inhibition after removal of the compound; that is, these inclusions changed from compact to network-like structures (Fig. 16), and the number of inclusion bearing cells increased. The yield of new infectious virus rose gradually, in parallel with the changes of morphological features of the inclusions.

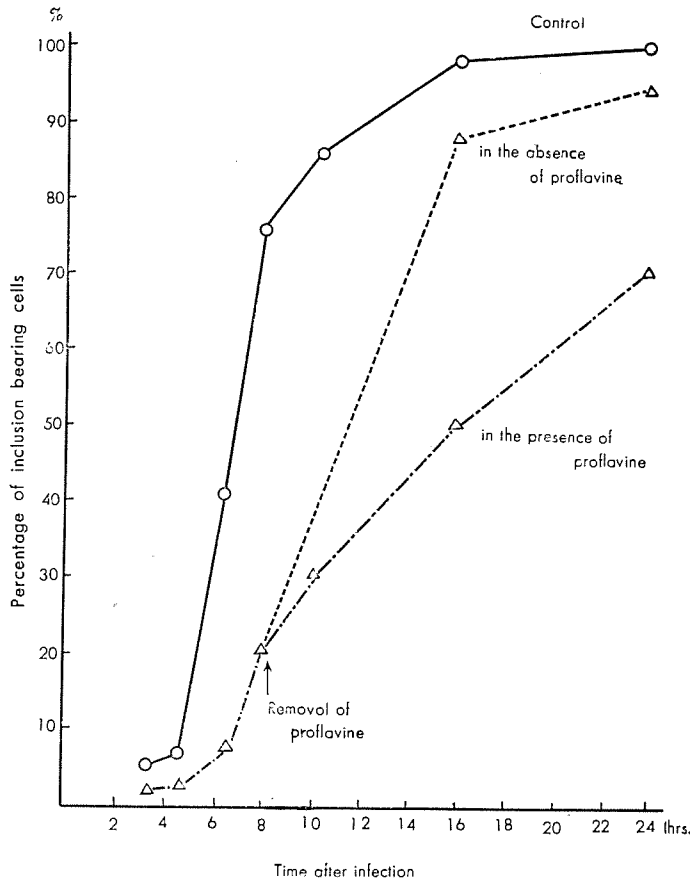


Fig. 10 Recovery of Virus Multiplication from Its Inhibited State by Removal of Proflavine
I. Recovery of Inclusion Formation

DISCUSSION

5, 6 - Dichloro - I - β - D - ribofuranosylbenzimidazole has a chemical structure analogous to 9-β-D-ribofuranosyladenine which is present in ribonucleic acid. It is known to affect specifically ribonucleic acid biosynthesis. However, there are only a few biochemical reports on the inhibitory action of DRB on ribonucleic acid biosynthesis (Tamm *et al.*, 1957b; Allfrey *et al.*, 1957).

On the other hand, there are many studies on chemotherapeutic effects on virus diseases. Tamm *et al.* worked on the inhibitory activity of various benzimidazole derivatives on the multiplication of influenza virus (1954a, 1954b, 1956a, 1956b) and vaccinia virus (1957a) in the chorioallantoic membrane *in vitro*, and of poliomyelitis virus in the HeLa cell culture (1957b).

It has been emphasized from their experimental results that DRB has a more selective inhibitory action on the multiplication of influenza virus than does vaccinia

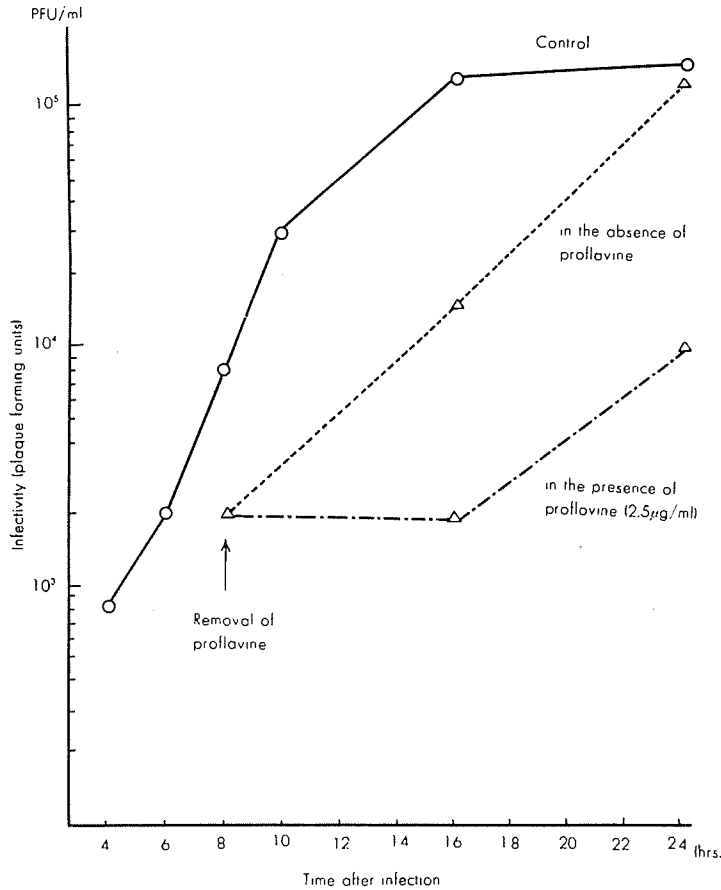


Fig. 11 Recovery of Virus Multiplication from Its Inhibited State by Removal of Proflavine
II. Recovery of the Yield of Infectious Virus Particles

virus, because the disturbance of cellular RNA metabolism induced by DRB presumably results in a direct suppression of the production of the specific RNA, which is an important constituent of influenza virus particles.

In our present work, aimed at determining the correlation between virus multiplication and cellular metabolism, we have observed a decrease in viral DNA synthesis, probably due to some metabolic disturbance of RNA, induced by the presence of a low concentration of DRB, which has little significant effect on cell proliferation or on the multiplication of the virus after its adsorption.

Similar phenomenon could also be seen in experiments with RNase under the same conditions. These phenomena were not observed by Tamm, and suggest that normal RNA metabolism may be essential for synthesis of the components from which progeny virus particles are made, regardless of the sort of nuleic acid present in the virus particles.

On the other hand, it was also observed in our experiments that the multiplication of virus during the postadsorptive phase was considerably suppressed by a high concentration of DRB. This is in support of Tamm's report concerning the inhibitory action of DRB on the multiplication of vaccinia virus.

The effect of RNase on virus growth has been investigated on tobacco mosaic virus by Casterman and Jeener (1957) and Benda (1958), and on influenza virus by Le Clerc (1956). Le Clerc plausibly considers that RNase may act directly on naked RNA of the parental virus inside the cells within 2 hours after the virus adsorption, resulting in diminished production of progeny.

However, our results indicate that a metabolic disturbance of RNA is likely to be responsible for a significant inhibition of virus growth, even if the virus particles are composed of DNA. Our finding is rather in support of the hypothesis that the virus first induces the formation of new protein in the infected cells, which is necessary for the formation of virus precursors: that a distubance of cellular RNA metabolism would result in the suppression of this new protein synthesis which would, in turn, inhibit virus synthesis.

Brachet (1955 1956, 1959) has reported several observations on the action of RNase on living cells using Amoeba and onion root-tips cells. He concluded that RNase easily forms a complex with soluble RNA rather than with other cell fraction, and this complex formation inhibits RNA and protein metabolism. At a later stage, RNA combined with RNase is broken down in the usual way.

His consideration is in agreement with our interpretation of the suppressive action of RNase on the multiplication of DNA containing virus. The close relationship between cellular RNA metabolism and the propagation of DNA containing virus has been done studied by several investigators. Work has been done on T_2 phage infected bacteria by Volkin and Astrachan (1958, 1959), and on Hela cells infected with vaccinia virus by Joklik (1959a, b, c), and Nishimura and Tagaya (1959), with the aid of radioisotopes.

Chang (1959) has also established a close relationship between cellular RNA metabolism and the synthesis of vaccinia, coxsackie and poliomyelitis virus using bicarbonate-depleted HeLa cell cultures.

Proflavine is generally known to suppress protein synthesis. We found in the present experiments that the mode of action of this compound differed from that of DRB in the process of virus growth. The action of proflavine at a low level is likely to interfere with some reaction in the processes leading to the production of infectious virus particle from virus precursors and other viral components rather than in the processes initiating their formation. In other words, it seems to interfere with the maturation process of the progeny virus.

This finding seems to support the idea of De Mars (1955) deduced from the suppressive effect of proflavine on phage infected bacteria (Foster, 1948). Similar observations have been made with other animal viruses (Franklin, 1958; Ledinko, 1958, 1959).

However, the possibility that proflavine treatment may also interfere with the synthesis of some as yet unrecognized virus constituents in the infected cells can not be neglected.

Since virus propagation, previously inhibited by a toxic concentration of proflavine, can be resumed on removal of proflavine, it may be that viral maturation may involve enzymes and steps not involved in normal host cell metabolism.

However, the step in virus synthesis which is most vulnerable to DRB is probably essential for virus reproduction and appears to be intimately connected with metabolism of the normal cell.

Finally, we propose the scheme for the action of the compounds used as shown in Fig. 12.

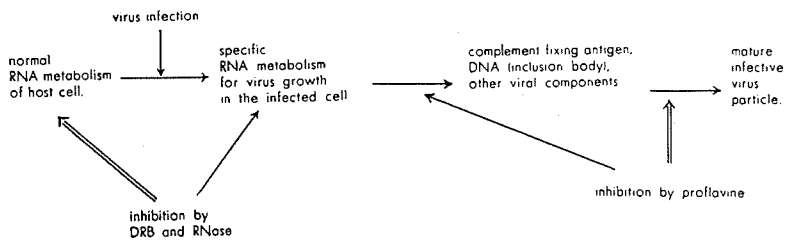


Fig. 12 Scheme for the Action of the Compounds Used

REFERENCES

Allfrey, V. G., Mirsky, A. E. and Osawa, S. (1957). Protein synthesis in isolated cell nuclei. *J. Gen. Physiol* **40,** 451–490.

Astrachan, L. and Volkin, E. (1958). Properties of ribonucleic acid turnover in T_2 infected Escherichia coli. *Biochim. Biophys. Acta.* **29,** 536–544.

Astrachan, L. and Volkin, E. (1959). Effect of chloramphenicol on ribonucleic acid metabolism in T_2 infected *Escherichia coli.* *Biochim. Biophys. Acta.* **32,** 449–456.

Benda, G. T. A. (1958). The introduction of tobacco mosaic virus into single hair cells of Nicotiana langsdorffii leaves. II. The effect of ribonuclease. *Virology* **6,** 718–724.

Brachet, J. (1955). Action of ribonuclease and ribonucleic acid on living Ameba. *Nature* **175,** 851–853.

Brachet, J. (1956). The mode of action of ribonuclease on living root-tips. *Biochim. Biophys. Acta.* **19,** 583.

Brachet, J. (1959). New observation on the mode of action of ribonuclease on living root-tips. *Biochim. Biophys. Acta.* **35,** 580–581.

Caterman, C. Hamers-, and Jeener, R. (1957). An initial ribonuclease-sensitive phase in the multiplication of tobacco mosaic virus. *Virology.* **3,** 197–206.

Chang, R. S. (1959). Participation of bicarbonate in RNA and protein synthesis as indicated by virus propagation in human cells. *J. Exptl. Med.* **109,** 229–238.

De Mars, R. I. (1955). The production of phage related materials when bacteriophage development is interrupted by proflavine. *Virology* **1,** 83–99.

Foster, R. A. C. (1948). An analysis of the action of proflavine on bacteriophage growth. *J. Bacteriol.* **56,** 795–809.

Franklin, R. M. (1958). The synthesis of fowl plague virus products in proflavine inhibited tissue culture system. *Virology* **6,** 525–539.

Furusawa, E., Kameyama, S., Kim, S., Iwa, K., Oketani, J. and Miyagawa, T. (1958). Multiplication of ectromelia virus in culture of strain L cell. *Virus* **8,** 499–503.

Furusawa, E., Hagiwara, K. and Kamahora, J. (1956). I. The effect of antibody on

intracellular virus multiplication. *Med. J. Osaka Univ.* **7**, 551–560.

Hagiwara, K. and Kamahora, J. (1956). The mechanism of the growth of ectromelia virus propagated in the ascites tumor cells. III. Morphological observation on the growth cycle *in vivo* and *in vitro*. *Med. J. Osaka Univ.* **7**, 571–588.

Ikegami, N., Kato, S. and Kamahora, J. (1959). A study on the inhibitory activity of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) and proflavine on the one-step growth cycle of the mouse pox virus (ectromelia virus) in L cells. *Biken's J.* **2**, 215–219.

Joklik, W. K. (1959a). Some aspects of the effect of infection of HeLa cells with vaccinia virus on the metabolism of RNA. *Biochemistry of Viruses. In proceeding of the Fourth International Congress of Biochemistry*, pp. 233–236. Vienna.

Joklik, W. K. and Rodrick, McN. (1959b). Biochemical studies on vaccinia virus in cultured cells. I. Incorporation of adenine-8-C^{14} into normal and infected cells. *Virology* **9**, 396–416.

Joklik, W. K. (1959c). Biochemical studies on vaccinia virus in cultured cells. II. Localization of adenine-8-C^{14} incorporated in the course of infection. *Virology* **9**, 417–424.

Kamahora, J., Kato, S., Baba, E. and Hagiwara, K. (1955). Studies on the inclusion bodies of fowlpox virus. *Med. J. Osaka Univ.* **6**, 745–754.

Kamahora, J., Ikegami, N., Kameyama, S. and Takahashi, M. (1957). The effect of infection of ectromelia virus on mitosis of Ehrlich ascites tumor cells. *Med. J. Osaka Univ.* **7**, 687–699.

Kamahora, J., Toyoshima, K., Nii, S. and Kato, S. (1959). A study on the number of infectious particles necessary for the "B" type inclusion formation. *Biken's J.* **2**, 345–352.

Kameyama, S., Takahashi, M., Kato, S., Toyoshima, K. and Kamahora, J. (1959). Studies on the inclusion bodies of ectromelia virus using fluorescent antibody technique. *Biken's J.* **2**, 341–344.

Kato, S. (1955). Studies on the inclusion bodies of ectromelia virus propagated in the ascites tumor cell. *Virus* **5**, 111–118.

Kato, S., Hagiwara, K. and Kamahora, J. (1955). The mechamism of the growth of ectromelia virus propagated in the ascites tumor cells. *Med. J. Osaka Univ.* **6**, 39–50.

Kato, S. and Cutting, W. (1959). A study of the inclusion bodies of rabbit myxoma and fibroma virus and a consideration of the relationship between all pox virus inclusion bodies. *Stanford Medical Bulletin* **7**, 34–45.

Kato, S., Takahashi, M., Kameyama, S. and Kamahora, J. (1959). A study on the morphological and cytoimmunological relationship between the inclusions of variola, cowpox, rabbit pox, vaccinia (variola origin) and vaccinia IHD and a consideration of the term "Giarnieri body". *Biken's J.* **2**, 353–363.

Kato, S., Kameyama, S. and Kamahora, J. (1960). Autoradiography with tritium-labeled thymidine of pox group viruses and human amnion cells system in tissue *cultures*. *Biken's J.* **3**, 135–138.

Le Clerc, J. (1956). Action of ribonuclease on the multiplication of the influenza virus. *Nature* **177**, 578–579.

Ledinko, N. (1958). Production of non-infectious complement fixing poliovirus particles in HeLa cells treated with proflavine. *Virology* **6**, 512–524.

Ledinko, N. (1959). Blocking of neutralizing antibody by non-infective poliovirus from proflavine-treated HeLa cells. *Virology* **8**, 536–539.

Marshall, J. D., Eveland, V. C. and Smith, C. W. (1958). Superiority of fluorescein isothiocyanate (Riggs) for fluorescent antiboy technique with a modification of its applicaion. *Proc. Soc. Exptl. Biol. med.* **98**, 898–900.

Nii, S. (1959). Plaque formation and some cytopathogenic characteristics of ectromelia

virus (Hampstead strain) and vaccinia virus (IHD strain) on monolayers. *Biken's J.* **2**, 195–206.

Nishimura, C. and Tagaya, I. (1959). Metabolic alterations of nucleotides and nucleic acids in HeLa cells infected with vaccinia virus. *Jap. J. Med. Sci. Biol.* **12**, 405~420.

Takahashi, M., Kato, S., Kameyama, S. and Kamahora, J. (1959). Studies on the inclusion bodies of rabbit myxoma virus with fluorescent antibody technique. *Biken's J.* **2**, 333–340.

Tamm, I. and Tyrrell, D.A.J. (1954a). Influenza virus multiplication in the chorio-allantoic membrane *in vitro*: Kinetic aspects of inhibition by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. *J. Exptl. Med.* **100**, 541–562.

Tamm, I., Folkers, K., Shunk, C. H. and Horsfall, F. L., jr. (1954b). Inhibition of influenza virus multiplication by N-glycosides of benzimidazoles. *J. Exptl. Med.* **99**, 227–250.

Tamm, I., Folkers, K. and Shunk, C. H. (1956a). High inhibitory activity of certain halogenated ribofuranosylbenzimidazole on influenza B virus multiplication. *J. Bacteriol.* **72**, 54–58.

Tamm, I., Folkers, K. and Shunk, C. H. (1956b). Certain benzimidazoles, benzens and ribofuranosylpurines as inhibitors of influenza B virus multiplication. *J. Bacteriol.* **72**, 59–64.

Tamm, I. and Overman, J. R. (1957a). Relationship between structure of benzimidazole derivatives and inhibitory activity on vaccinia virus multiplication. *Virology* **3**, 185–196.

Tamm, I. and Nemes, M. M. (1957b). Glycosides of chlorobenzimidazoles as inhibitors of poliovirus multiplication. *Virology* **4**, 483–498.

Weller, T. H. and Coons, A. H. (1954). Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*. *Proc. Soc. Exptl. Biol. Med.* **86**, 789–794.

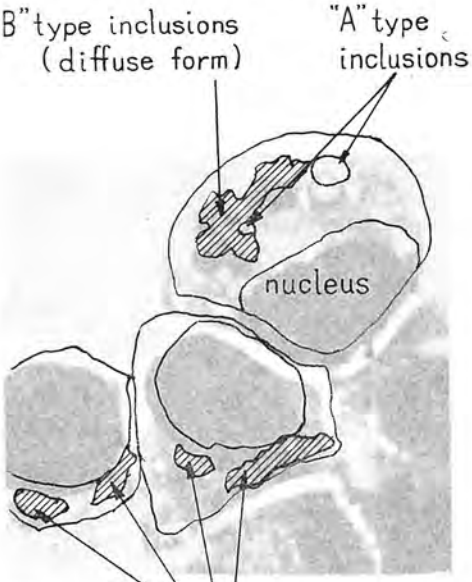


Fig. 13. Most of the "B" type inclusions in the proflavine treated infected cells had a diffuse form 16 hours after virus infection.

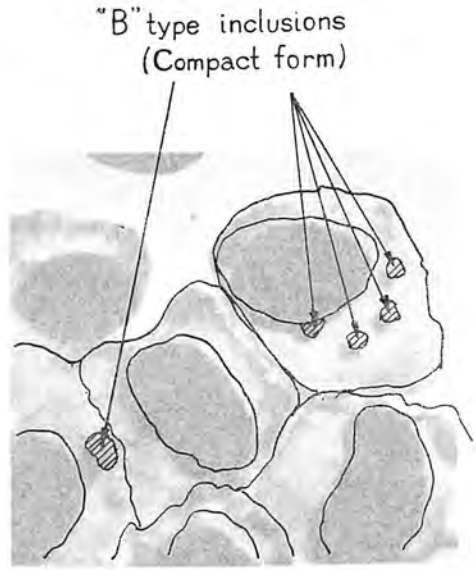


Fig. 14. The "B" type inclusions in the cells post-treated with 2.5 μg of proflavine were small and compact 16 hours after infection.

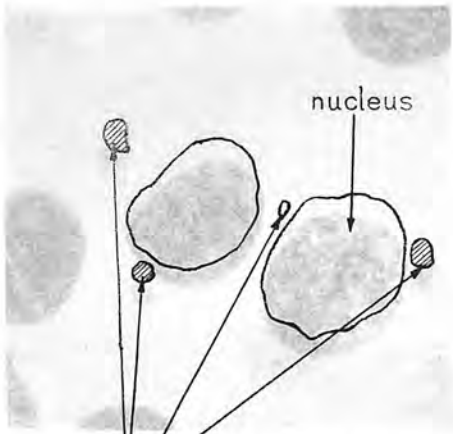


Fig. 15. Proflavine treated "B" type inclusions shown in this figure gave the same degree of Feulgen reaction as the infected cells.

Feulgen reaction positive

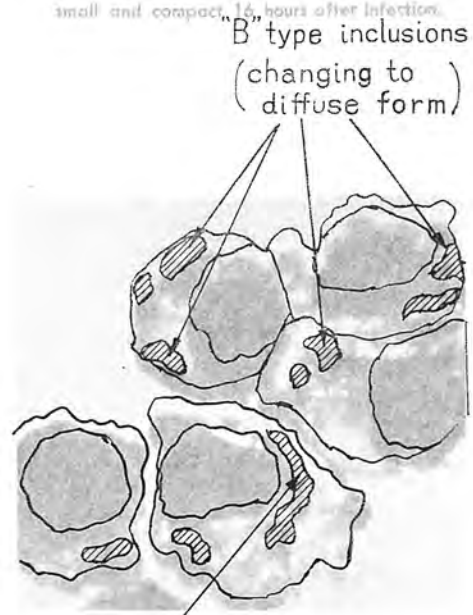


Fig. 16. The "B" type inclusions in 8 hour proflavine treated cells, after removal of proflavine, changed from a compact form to a diffuse form. This figure shows the inclusions 8 hours after removal of proflavine.

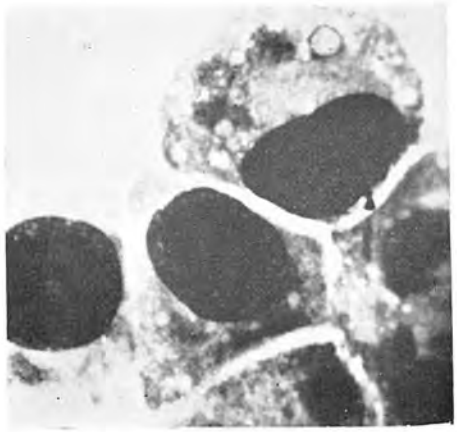


Fig. 13. Most of the "B" type inclusions in the untreated infected cells had a diffuse form 16 hours after virus infection.

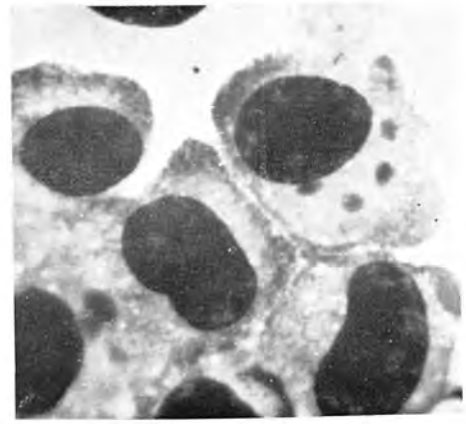


Fig. 14. The "B" type inclusions in the cells post-treated with 2.5 μg of proflavine were small and compact 16 hours after infection.

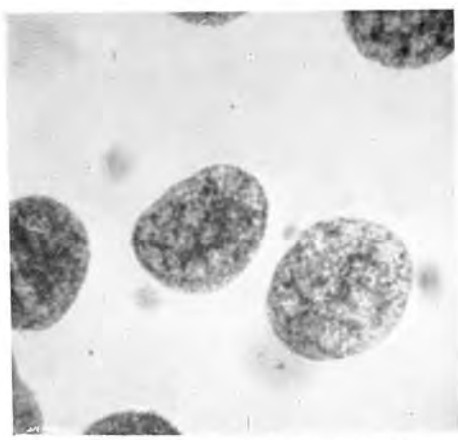


Fig. 15. Proflavine treated "B" type inclusions shown in this figure gave the same degree of Feulgen positive reaction as untreated cells.

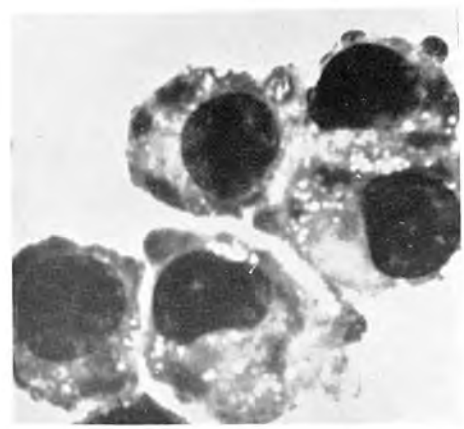


Fig. 16. The "B" type inclusions in 8 hour proflavine treated cells, after removal of proflavine, changed from a compact form to a network-like structure. This figure shows the inclusions 8 hours after removal of proflavine.