

## Rifampicin: a Specific Inhibitor of Vaccinia Virus Assembly

by

BERNARD MOSS  
EDITH N. ROSENBLUM  
EHUD KATZ

Laboratory of Biology of Viruses,  
National Institute of Allergy  
and Infectious Diseases

PHILIP M. GRIMLEY

Laboratory of Pathology,  
National Cancer Institute,  
Bethesda, Maryland 20014

Rifampicin prevents the assembly of DNA and proteins into mature virus particles. The block, which occurs at a stage in the formation of the viral envelope, can be rapidly reversed by removal of the drug.

RIFAMPICIN and related rifamycin derivatives bind to the RNA polymerase of bacterial cells preventing both bacterial growth and phage replication<sup>1-4</sup>. Rifampicin also inhibits the replication of vaccinia virus and adenovirus in mammalian cells<sup>5,6</sup>. Because this class of antibiotics has little effect on mammalian RNA polymerase<sup>7-9</sup>, it was initially suggested that rifampicin might act directly on viral enzymes concerned with RNA synthesis. Subsequent experiments, however, indicated that vaccinia RNA is synthesized in the presence of rifampicin (refs. 10 and 11; B. R. McAuslan, personal communication). We found<sup>10</sup> that concentrations of rifampicin which completely prevented virus growth did not inhibit the RNA polymerase activity associated with the mature virus particle or virus core. Furthermore, rifampicin did not inhibit the intracellular synthesis of "early" viral messenger RNA, which is transcribed from parental DNA templates, or "late" viral messenger RNA, which is transcribed after the synthesis of progeny DNA<sup>10</sup>. The two classes of RNA were distinguished by their sedimentation properties as well as by their time of synthesis<sup>10</sup>. We found, moreover, that specific "early" and "late" viral proteins, identified by immunodiffusion, immunoelectrophoresis and disc gel electrophoresis, and synthesized in the presence of rifampicin<sup>10</sup>.

We suggested<sup>10</sup> that either rifampicin inhibits the synthesis of only certain specific vaccinia RNA species or acts in a novel manner not predicted from studies with bacterial systems. Evidence that rifampicin prevents the assembly of DNA and proteins into mature virus particles, and that the block occurs at a stage in the formation of the viral envelope, will now be presented.

### Viral DNA Synthesis

Vaccinia DNA is synthesized in HeLa cells during the interval from 2 to 5 h after infection<sup>12,13</sup>. Formation of infectious virus can be detected by 5 h and a nearly complete yield of virus is obtained at 18 h. Because vaccinia virus replicates within the cytoplasm of the cell, a technique is thus available for specifically following viral DNA synthesis<sup>13</sup>. Infected cells are pulsed with a radioactively labelled DNA precursor and then disrupted by careful mechanical homogenization. Nuclei are removed by centrifugation and the quantity of radioactively labelled DNA in the cytoplasmic fraction is measured. This method was used in an experiment which showed that the time course and extent of viral DNA

synthesis are only slightly altered when HeLa cells are infected in the presence of 100 µg of rifampicin per ml. (Fig. 1). This finding is in agreement with that of Ben-Ishai *et al.*<sup>11</sup>.

### Fate of Viral DNA

Joklik and Becker<sup>13</sup> followed the fate of newly synthesized vaccinia DNA during the normal replicative cycle. At about 5 h after infection, the viral DNA in the crude cytoplasmic fraction became progressively more resistant to the action of pancreatic deoxyribonuclease.

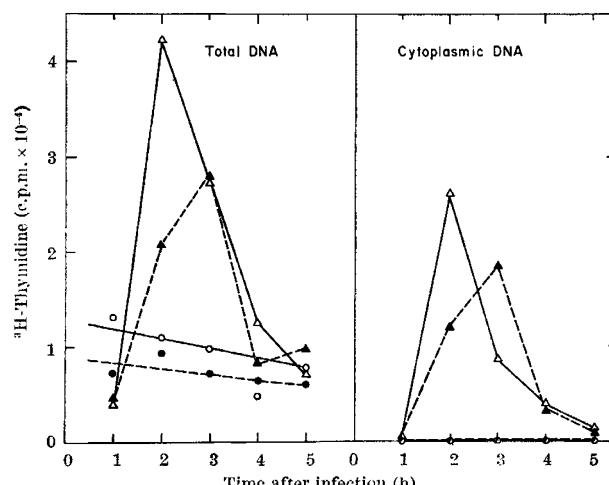


Fig. 1. Effect of rifampicin on viral DNA synthesis. HeLa cells were suspended at a concentration of  $4 \times 10^6$ /ml. in Eagle's medium<sup>16</sup> containing 5 per cent horse serum. Rifampicin was added (100 µg/ml.) and after 10 min at 37° C., the cells were infected with purified vaccinia virus, strain WR, at a multiplicity of 30 plaque forming units (p.f.u.) per cell. After 30 min the cell suspension was diluted, with medium containing rifampicin, to a concentration of  $4 \times 10^4$  cells/ml. Duplicate cultures of uninfected and infected cells without rifampicin were treated similarly. At intervals, 20 ml. portions of the cell suspension were incubated with 6.25 µCi of <sup>3</sup>H-thymidine (14.4 Ci/mole, New England Nuclear) for 10 min. The pulses were terminated by pouring the cells over frozen, crushed saline. The washed cells were then resuspended in 1 ml. of 10 mM Tris, 10 mM NaCl, 1.5 mM MgCl<sub>2</sub>, pH 7.8 (RSB), and broken with a Dounce homogenizer; nuclei were removed by centrifugation at 200g for 2 min. Radioactivity in the total cellular and cytoplasmic trichloroacetic acid precipitable material was measured by liquid scintillation counting. △—△, Infected and without rifampicin; ▲—▲, infected and with rifampicin; ○—○, uninfected and without rifampicin; ●—●, uninfected and with rifampicin.

Development of deoxyribonuclease resistance depended on protein synthesis and presumably resulted from "coating" of the DNA with proteins.

We have followed the fate of newly synthesized vaccinia DNA made in the presence of rifampicin using deoxyribonuclease digestion after sucrose gradient centrifugation. Without the drug, DNA labelled with  $^3\text{H}$ -thymidine between 2 and 3 h after infection was found 17 h later in both a deoxyribonuclease digestible form which remained at the top of the sucrose gradient and in a resistant band coincident with infectious virus (Fig. 2). In the presence of rifampicin, no virus band was detected and the radioactively labelled DNA remained in a deoxyribonuclease sensitive form near the top of the gradient (Fig. 2).

### Virus from DNA made in Presence of Rifampicin

The next object was to determine whether DNA made in rifampicin treated cells represented complete and functional viral genomes. The first experiment showed that DNA, labelled with  $^3\text{H}$ -thymidine between 1.5 and 3 h after infection, was converted to a deoxyribonuclease resistant form after the removal of rifampicin (Fig. 3). This labelled DNA was also "chased" into particles which, after purification, sedimented with carrier virus in sucrose gradients (Fig. 4). A final experiment, summarized in Table 1, indicated that a full yield of infectious virus was formed using the DNA made during 4.5 h of rifampicin treatment. Before removal of the rifampicin, 5-fluorodeoxyuridine (5-FUDR) was added, to prevent further DNA synthesis<sup>12</sup>.

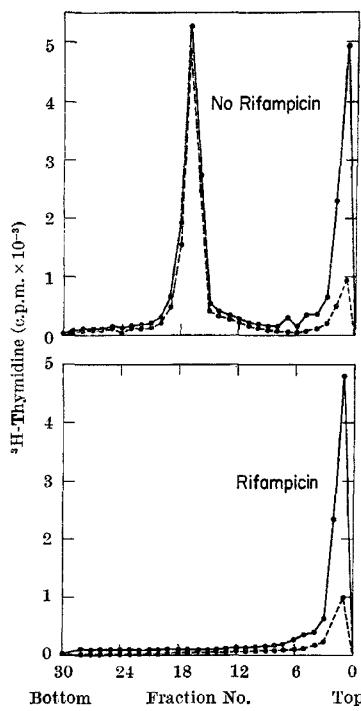


Fig. 2. Fate of viral DNA in the presence of rifampicin. HeLa cells, treated with rifampicin ( $100 \mu\text{g}/\text{ml}$ ), were incubated with  $^3\text{H}$ -thymidine ( $0.08 \mu\text{Ci}/\text{ml}$ ) between 2 and 3 h after infection. Then the cells were washed and resuspended in medium containing unlabelled thymidine ( $10^{-4} \text{ M}$ ) and rifampicin. After 17 h additional incubation the cells were washed in saline, resuspended in 1 mM sodium phosphate ( $\text{pH } 7.0$ ), disrupted with a Dounce homogenizer and layered directly on a 25 to 40 per cent linear sucrose gradient. After centrifugation in an SW 25.1 rotor at 15,000 r.p.m. ( $23,000g$ ) for 1 h, the gradient was passed through a continuous ultraviolet monitor and fractions collected. One portion of each fraction was directly precipitated with trichloroacetic acid and another was adjusted to 1 mM  $\text{MgCl}_2$  and incubated with pancreatic deoxyribonuclease ( $50 \mu\text{g}/\text{ml}$ ; Worthington) for 30 min at  $37^\circ\text{C}$  before precipitation. The fate of DNA in infected cells not treated with rifampicin was determined in the same manner. ●—●, Total; ●—●—●, deoxyribonuclease resistant.

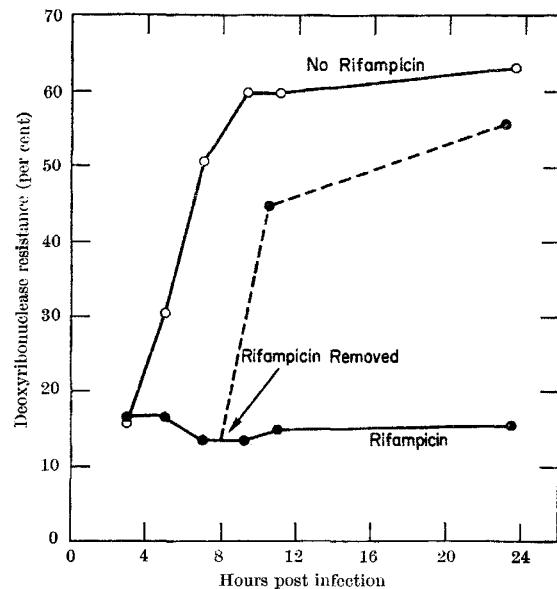


Fig. 3. Resistance to deoxyribonuclease after removal of rifampicin. HeLa cells, treated with rifampicin ( $100 \mu\text{g}/\text{ml}$ ), were incubated with  $^3\text{H}$ -thymidine ( $0.08 \mu\text{Ci}/\text{ml}$ ) from 1.5 to 3 h after infection. The cells were then washed and resuspended in medium containing unlabelled thymidine ( $10^{-4} \text{ M}$ ) and rifampicin. Samples were removed at intervals and the cytoplasmic fraction was prepared as in Fig. 1. One portion of the cytoplasm was directly precipitated with trichloroacetic acid and another was first digested with deoxyribonuclease as in Fig. 2. After liquid scintillation counting, the percentage of deoxyribonuclease resistant DNA was calculated. Changes in deoxyribonuclease resistance were also followed after removal of rifampicin and in a duplicate culture not treated at all with rifampicin.

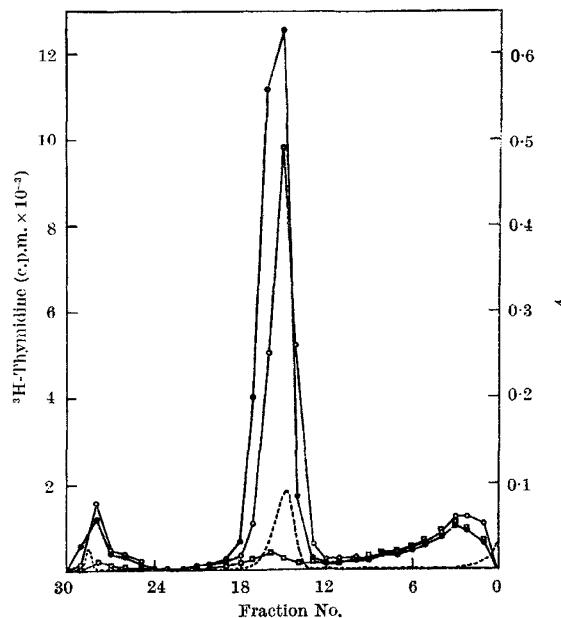


Fig. 4. Incorporation of DNA into virus particles after removal of rifampicin. HeLa cells treated with rifampicin ( $100 \mu\text{g}/\text{ml}$ ) were incubated with  $^3\text{H}$ -thymidine ( $0.2 \mu\text{Ci}/\text{ml}$ ) from 1 to 5 h after infection. Rifampicin was removed and the cells were resuspended in medium containing unlabelled thymidine ( $10^{-4} \text{ M}$ ) and incubated for an additional 15 h. Carrier unlabelled vaccinia virus was then added and the cells were frozen and thawed and further disrupted with sonic vibrations of 20 kHz for 45 s. Digestion with  $20 \mu\text{g}/\text{ml}$  each of pancreatic deoxyribonuclease and ribonuclease was carried out at  $37^\circ\text{C}$  for 30 min. The digest was then sedimented through 36 per cent sucrose in 1 mM Tris-HCl ( $\text{pH } 9$ ) in an SW 25.1 rotor at 15,000 r.p.m. ( $23,000g$ ) for 80 min. The pellets were resuspended and layered on a 25 to 40 per cent sucrose gradient. After 40 min at 13,500 r.p.m. ( $19,000g$ ) the gradient was passed through a continuous ultraviolet monitor and fractions were collected directly in scintillation vials. Incorporation of DNA into virus particles was also determined in a duplicate culture continuously treated with rifampicin and in a culture not treated at all with the drug. ●—●, No rifampicin; □—□, rifampicin continuously; ○—○, rifampicin from 1 to 5 h after infection; —, absorbance tracing of carrier virus.

### Synthesis of Viral Proteins

The failure of vaccinia DNA to become resistant to deoxyribonuclease while in the presence of rifampicin might result from the absence of structural proteins. Although we had previously shown<sup>10</sup> that certain "early" and "late" vaccinia viral proteins are made in rifampicin treated cells, the methods were capable of resolving only a small number of proteins. A more complete analysis of vaccinia proteins has now been made by pulse-labelling cells with <sup>14</sup>C-amino-acids for 1 h periods. Radioactively labelled proteins within the cytoplasm were solubilized with sodium dodecyl sulphate (SDS) before separation by polyacrylamide gel electrophoresis. The usual transition in vaccinia infected cells from the synthesis of host proteins to specific "early" viral and then "late" viral proteins<sup>14</sup> took place in the presence of rifampicin, although late protein synthesis declined more abruptly (Fig. 5). By contrast, 5-FUDR, an inhibitor of DNA synthesis, prevented the synthesis of late viral proteins (Fig. 5). This method demonstrated that a broad spectrum of viral proteins was synthesized in the presence of rifampicin; nevertheless the vaccinia genome is large enough to code for hundreds of polypeptides and it was impossible to conclude that all necessary viral proteins were made.

### Assembly of Virus in Presence of Cycloheximide

The experiments described showed that the complete viral genome and a large number of viral proteins were made in rifampicin treated cells. We therefore considered that this drug might act by preventing virus assembly. The object of subsequent experiments was to determine whether complete virus particles could be assembled, using only the proteins synthesized in the presence of rifampicin. The plan was to prevent further protein synthesis by adding cycloheximide before the removal of rifampicin. Virus assembly could then be determined by (a) the incorporation of previously formed radioactively labelled DNA and proteins into virus particles, (b) the increase in infectious virus titre, and (c) the identification of mature virus particles by electron microscopy.

For the first method we labelled the viral DNA made in rifampicin treated cells with <sup>14</sup>C-thymidine. Cycloheximide (300 µg/ml.) was added 8 h after infection and 10 min later rifampicin was removed. The incubation was continued in medium containing cycloheximide and at intervals samples were taken and mixed with carrier <sup>3</sup>H-thymidine labelled virus. Virus particles were purified by a procedure culminating in sucrose gradient centrifugation. The results, shown in Fig. 6, demonstrate that particles were assembled in the absence or presence of cycloheximide. Similar results were obtained in other experiments without carrier virus. An accurate time course of virus assembly was determined using the recovery of <sup>3</sup>H-thymidine labelled carrier virus which varied from 60 to 67 per cent in the different gradients (Fig. 7). Virus assembly proceeded at nearly the same rate for the first hour regardless of whether cycloheximide

Table 2. INCREASE IN INFECTIOUS VIRUS TITRE AFTER REMOVAL OF RIFAMPICIN

No.	Drug	Zero time	Virus titre 180 min
1	—	$3.6 \times 10^4$	$3.0 \times 10^5$
2	Cycloheximide	$3.2 \times 10^4$	$1.4 \times 10^6$
3	Rifampicin	$4.0 \times 10^4$	$4.2 \times 10^4$

Rifampicin was removed from Nos. 1 and 2 at 8 h after infection. Ten min before this, cycloheximide was added to No. 2. Rifampicin was not removed from No. 3. "Zero time" is 8 h after infection. The virus titre is expressed in plaque forming units. These samples were taken from the experiment described in Fig. 6.

was present. After this, the assembly rate was greater in the absence of cycloheximide presumably because of an expanding pool of viral proteins.

To interpret the previous experiment as indication that virus assembly occurred in the absence of further protein synthesis it was necessary to demonstrate that inhibition of protein synthesis was complete. A 98 per cent inhibition of amino-acid incorporation, produced by cycloheximide, was detected during the first 10 min interval after resuspension of cells in rifampicin-free medium. No radioactivity was detected in virus particles formed during the next 4 h and total protein synthesis was inhibited by 98 per cent during the entire period.

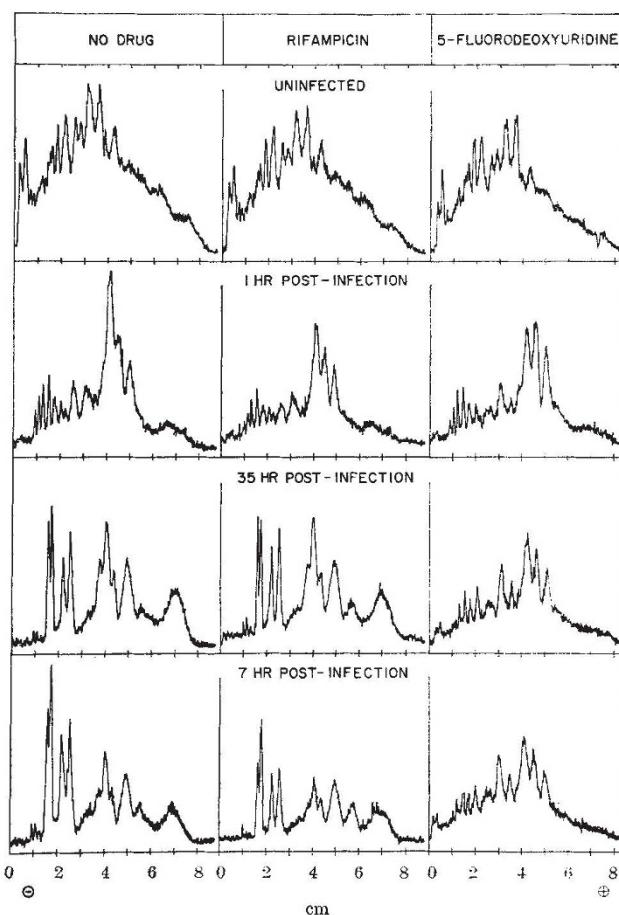


Fig. 5. Polyacrylamide gel electrophoresis. Replicate suspensions of HeLa cells were: (a) not treated with drugs; (b) treated with rifampicin (100 µg/ml.); and (c) treated with 5-FUDR ( $10^{-4}$  M). After 10 min, the cells were infected and at intervals 25 ml. portions were incubated with 12.5 µCi of mixed <sup>14</sup>C-amino-acids (103–330 mCi/mmol, Schwarz Bio Research) for 1 h. The labelling periods were terminated by rapid chilling and then the cells were washed in cold buffer and disrupted by homogenization. The proteins in the cytoplasmic fraction were solubilized by heating with SDS (2 per cent) and mercaptoethanol (1 per cent) at 100°C for 1 min. After dialysis (0.01 M sodium phosphate, pH 7.1, 0.1 per cent SDS, 0.1 per cent mercaptoethanol) for 20 h, the proteins were separated by polyacrylamide (7.5 per cent) gel electrophoresis at 3 mA per gel for 18 h<sup>17</sup>. The gels were immersed in trichloroacetic acid (20 per cent), stained with Coomassie blue (0.1 per cent in 10 per cent trichloroacetic acid), washed in acetic acid (7.5 per cent), sliced longitudinally, dried and exposed to X-ray film<sup>18</sup>. Tracings of the developed X-ray films were made with a Joyce-Loebl microdensitometer.

Table 1. FORMATION OF INFECTIOUS VIRUS WITH DNA MADE IN THE PRESENCE OF RIFAMPICIN

Drug treatment	0-4.5 h	4.5-20 h	Virus titre
5-FUDR	—	—	$1.5 \times 10^6$
—	5-FUDR	—	$4.4 \times 10^4$
Rifampicin	—	5-FUDR	$1.2 \times 10^6$
Rifampicin	5-FUDR	—	$1.6 \times 10^6$
Rifampicin	Rifampicin	—	$1.0 \times 10^4$

HeLa cells were treated with rifampicin (100 µg/ml.) and infected with vaccinia virus as in Fig. 1. At 4.5 h after infection 5-FUDR ( $10^{-4}$  M) was added. Rifampicin was removed 10 min later and the cells were incubated for an additional 15.5 h in medium containing 5-FUDR. Virus titres were determined by plaque assay on HeLa cell monolayers. The formation of infectious virus was also determined in cultures: not treated with drugs; treated continuously with 5-FUDR; and treated with 5-FUDR from 4.5 to 20 h. The latter control culture showed that 5-FUDR had no effect on virus maturation after the period of DNA synthesis.

In experiments similar to those described in Fig. 6, we found that radioactively labelled viral proteins made before the removal of rifampicin were "chased" into virus particles in the presence of cycloheximide. Furthermore, these particles were isolated and shown to contain all structural proteins previously identified by polyacrylamide gel electrophoresis<sup>14</sup>.

Virus particles formed in the presence of cycloheximide, after removal of rifampicin, were shown to be infectious (Table 2).

### Electron Microscopy

A block in the assembly of vaccinia virus, produced by rifampicin, was confirmed by electron microscopy. During the usual course of vaccinia infection, accumulation of a viroplasmic matrix in the host cell cytoplasm is the first ultrastructural evidence of virus development. By 3–4 h after infection, trilaminar unit membranes appear in the viroplasmic factory regions. These membranes are destined to form the vaccinia envelope. Initially they are observed in ultrathin sections as short circular arcs which are coated on the convex surface by a dense layer of spicules<sup>15</sup>. Gradually, the viral membranes arrange in uniform rings enclosing viroplasmic substance. In the presence of rifampicin, the trilaminar membranes, which

develop at 4–8 h after infection, lack a defined spicule layer and fail to form rigid arcs. In profile, these membranes are grouped in large irregular circles, girdling domains of viroplasm which appear more electron dense than the surrounding matrix and contain material sufficient to form several virus particles (Fig. 8, top). Within 10 min after removal of the rifampicin, the membrane circles are converted into uniform arcs which now exhibit a regular external spicule layer (Fig. 8, middle). These coated membranes are ultrastructurally similar to the envelopes of immature particles which are observed in the normal replication cycle, and some membranes appear to pass slowly through the typical stages of viroplasmic enclosure and internal organization to form virions (Fig. 8, bottom). Both the rapid and slow transitional stages were observed by electron microscopy in the presence of cycloheximide. We believe the viral membranes which develop in the presence of rifampicin are precursors of envelope membrane because of their unit structure and rapid conversion into coated membranes typical of immature vaccinia envelopes. A detailed study of the formation, structure, maturation and chemical composition of the precursor membranes is in progress.

From biochemical and electron microscopic studies, we conclude that rifampicin interferes with the assembly of

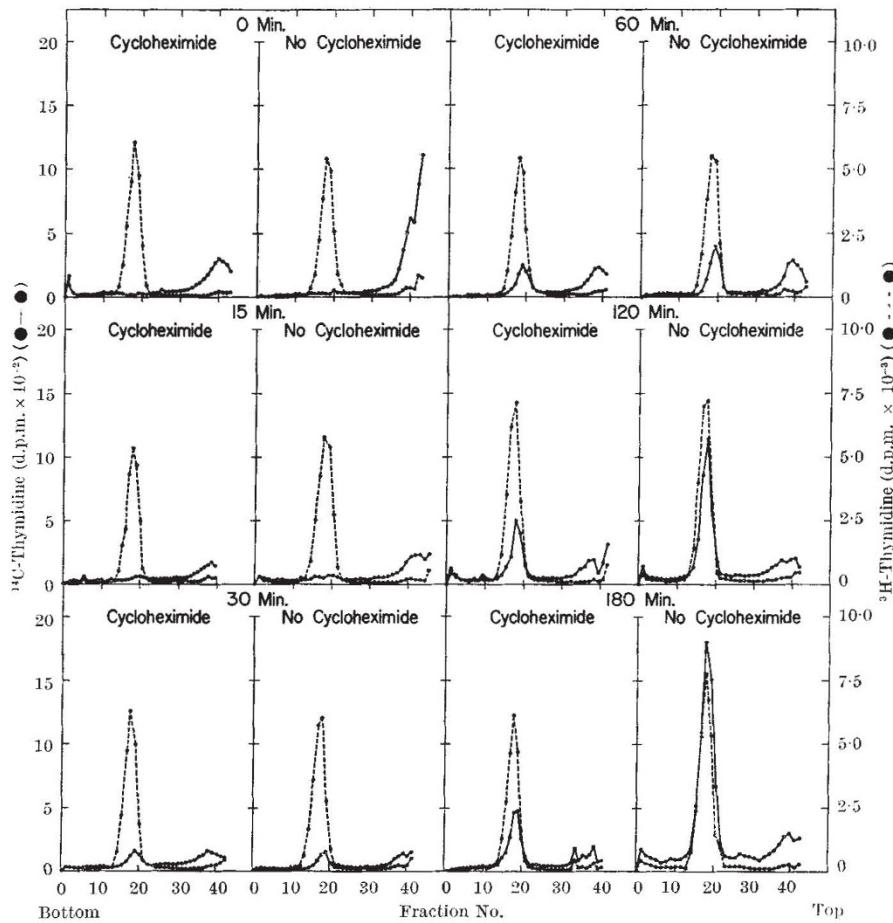


Fig. 6. Virus assembly in the presence of cycloheximide after the removal of rifampicin. Viral DNA synthesized in the presence of rifampicin (100 µg/ml.) was labelled with <sup>14</sup>C-thymidine (0.2 µCi/ml.; 52.8 mCi/mmol; New England Nuclear) from 1 to 4 h after infection. The cells were then washed and resuspended in medium containing unlabelled thymidine (10<sup>-6</sup> M) and rifampicin. At 8 h after infection, cycloheximide (300 µg/ml.) was added to one portion of the cells. Ten min later, the cells were washed in cold medium without rifampicin and containing cycloheximide. The incubation was continued and at intervals portions were removed. Carrier <sup>3</sup>H-thymidine labelled virus was added and purification was carried out as in Fig. 4 except that an SW 50.1 rotor was used and the final centrifugation was at 13,000 r.p.m. (15,800g) for 35 min. Fractions (two drops), collected by piercing the bottoms of the tubes, were dissolved in a toluene scintillator solvent (Liquifluor, New England Nuclear) containing 'Biosolv 3' (Beckman). A control, in which cycloheximide was not added at the time of removal of rifampicin, was treated in the same manner.

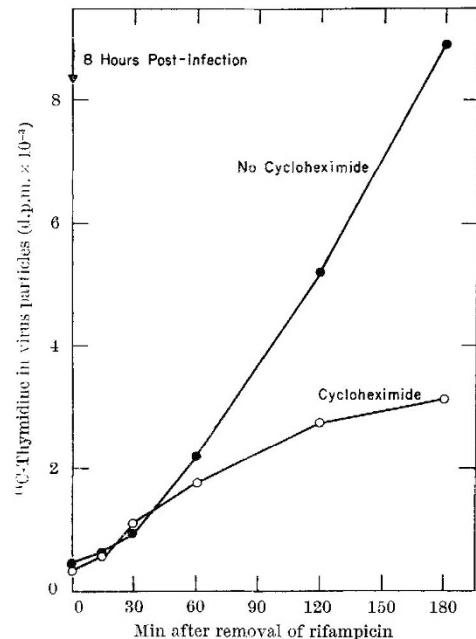


Fig. 7. Kinetics of virus assembly. The quantity of virus assembled was determined from each gradient in Fig. 6 by adding all of the  $^{14}\text{C}$  radioactivity coincident with the  $^3\text{H}$ -carrier virus peak. These values were then corrected for the recovery of  $^3\text{H}$ -virus in the same gradient. (Note that although the zero time points are slightly above the origin, no detectable virus peaks were present at this time as seen in Fig. 6.)

DNA and proteins into virus particles at a stage in the formation of the virus envelope. We suspect that other effects of rifampicin such as the more abrupt decline in late viral protein synthesis are secondary, perhaps resulting from the accumulation of unassembled structural proteins.

We thank Dr R. B. Nolan of the Dow Chemical Co. for a sample of rifampicin with a stated purity of 992.5  $\mu\text{g}/\text{mg}$ .

*Note added in proof.* Recently, McAuslan reported (*Biochem. Biophys. Res. Commun.*, **37**, 289; 1969) that vaccinia RNA polymerase activity does not increase in cells infected in the presence of rifampicin. This finding, which we have confirmed, can be related to our present work in two alternative ways: (1) the postulated binding of rifampicin to RNA polymerase also prevents completion of the viral envelope and virus assembly, or (2) the primary action of rifampicin is to block virus assembly at a stage in the formation of the viral envelope and consequently RNA polymerase is not incorporated into particles. At present there is no reason to prefer the former, except by analogy with bacterial systems, because active vaccinia RNA polymerase is a component of the virus core and has never been detected in a nonparticulate form.

Received October 27, 1969.

- <sup>1</sup> Wehrli, W., Knusel, F., Schmid, K., and Staehelin, M., *Proc. US Nat. Acad. Sci.*, **61**, 667 (1968).
- <sup>2</sup> Sippel, A., and Hartmann, G., *Biochim. Biophys. Acta*, **157**, 218 (1968).
- <sup>3</sup> Gelduscheck, E. P., and Sklar, J., *Nature*, **221**, 833 (1969).
- <sup>4</sup> Haselkorn, R., Vogel, M., and Brown, R. D., *Nature*, **221**, 836 (1969).
- <sup>5</sup> Heller, E., Argaman, M., Levy, H., and Goldblum, N., *Nature*, **222**, 273 (1969).
- <sup>6</sup> Subak-Sharpe, J. H., Timbury, M. C., and Williams, J. F., *Nature*, **222**, 341 (1969).
- <sup>7</sup> Wehrli, W., Nuesch, J., Knusel, F., and Stachelin, M., *Biochim. Biophys. Acta*, **157**, 215 (1968).
- <sup>8</sup> Umezawa, H., Mizuno, S., Yamazaki, H., and Nitta, K., *J. Antibiotics*, **21**, 234 (1968).
- <sup>9</sup> Jacob, S. T., Sajdel, E. M., and Munro, H. N., *Biochem. Biophys. Res. Commun.*, **32**, 831 (1968).
- <sup>10</sup> Moss, B., Katz, E., and Roseblum, E. N., *Biochem. Biophys. Res. Commun.*, **36**, 858 (1969).
- <sup>11</sup> Ben-Ishai, Z., Heller, E., Goldblum, N., and Becker, Y., *Nature*, **224**, 29 (1969).
- <sup>12</sup> Salzman, N. P., *Virology*, **10**, 150 (1960).
- <sup>13</sup> Joklik, W. K., and Becker, Y., *J. Mol. Biol.*, **10**, 452 (1964).

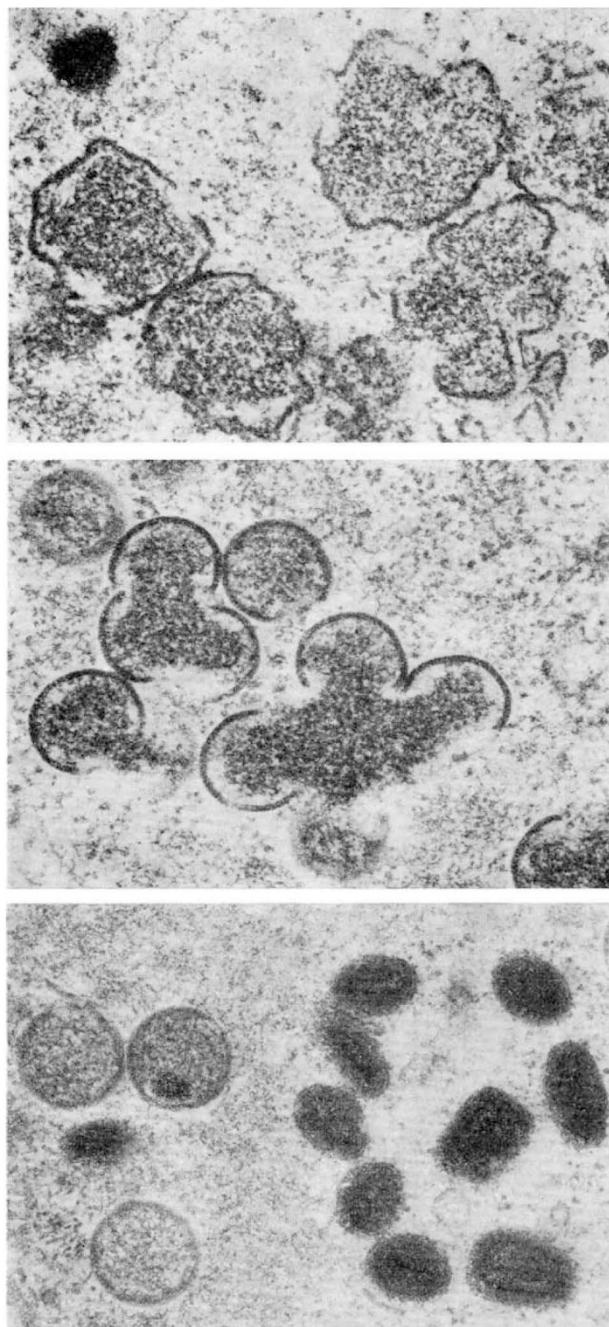


Fig. 8. Electron microscopy. HeLa cells treated with rifampicin (100  $\mu\text{g}/\text{ml}$ ) were infected with vaccinia virus. After 8 h, the drug was removed and the incubation continued. At intervals, portions were taken and cell pellets were formed by centrifugation at 800*g*. These were fixed immediately in chilled 3 per cent glutaraldehyde and then dehydrated and embedded in 'Epon' according to reported procedures<sup>12</sup>. Ultra-thin sections were stained with uranyl acetate and lead citrate before examination in a Perkin-Elmer Hitachi HU-11E microscope at 50 or 75 kV. Top: rifampicin effect; regions of dense viroplasm bounded by discontinuous segments of trilaminar unit membrane ( $\times 43,000$ ). Middle: 10 min after removal of rifampicin; viral membranes converted to uniform arcs with an outer dense coat ( $\times 40,000$ ). Bottom: 4 h after removal of rifampicin; progressive maturation, immature particles at left; cluster of virions at right ( $\times 39,000$ ).

<sup>14</sup> Moss, B., and Salzman, N. P., *J. Virol.*, **2**, 1016 (1968).

<sup>15</sup> Dales, S., and Mosbach, E. H., *Virology*, **35**, 564 (1968).

<sup>16</sup> Eagle, H., *Science*, **130**, 432 (1959).

<sup>17</sup> Maizel, jun., J. V., in *Fundamental Techniques of Virology* (edit. by Habel, K., and Salzman, N. P.), 334 (Academic Press, New York, 1969).

<sup>18</sup> Fairbanks, jun., G., Levinthal, C., and Reeder, R. H., *Biochem. Biophys. Res. Commun.*, **20**, 393 (1965).

<sup>19</sup> Grimley, P. M., Deftoes, L. J., Weeks, J. R., and Rabson, A. S., *J. Nat. Cancer Inst.*, **42**, 663 (1969).