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Inhibition of Poxvirus Maturation by Rifamycin Derivatives and Related Compounds

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The effect of a number of rifamycin derivatives and related compounds on the reversibility of the rifampin-induced virus maturation block was studied by using BHK-21 cells infected with vaccinia virus. All of the derivatives of 3-formyl rifamycin SV maintained this block, the required concentration varying from 100 to 1,000 μg/ml. These compounds vary only in the nature of the side-chain attached to the 3C atom on the naphthohydroquinone moiety; no obvious correlation between the nature of this side-chain and antiviral activity was found. Streptovaricin complex and tolypomycin R also maintained the maturation block; tolypomycin also produced marked alterations in the appearance of the viroplasm contained in rifampin-induced inclusions and immature virus particles.

Rifampin, a semisynthetic antibiotic of the ansamycin group, specifically inhibits the growth of vaccinia virus, preventing the formation of immature virus particles (6, 11, 13, 19). This block is rapidly reversed after removal of the drug in the presence of inhibitors of protein, deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) synthesis (10–13), strongly suggesting that rifampin does not inhibit the synthesis of immature particle components but that it inhibits their assembly. Rifampin is known to bind firmly and specifically to bacterial DNA-dependent RNA polymerase, with subsequent inhibition of transcription (3, 18, 22), but extensive studies with vaccinia virus have failed to demonstrate a similar interaction, the activity of the virion DNA-dependent RNA polymerase and the synthesis in infected cells of viral DNA, RNA, and early and late proteins not being prevented by concentrations of rifampin sufficient to inhibit virus growth completely (1, 2, 8, 10, 19).

Rifampin is synthesized by the condensation of 3-formyl rifamycin SV with 1-amino 4-methyl piperazine (9). Studies with these compounds have shown that the antiviral activity of rifampin resides in the rifamycin moiety of the compound (Follett and Pennington, *in press*). In this paper we report the results of studies on the antiviral activity of several semisynthetic rifamycin derivatives (9, 16) and two naturally occurring ansamycin antibiotics, streptovaricin (15) and tolypomycin-R-(7).

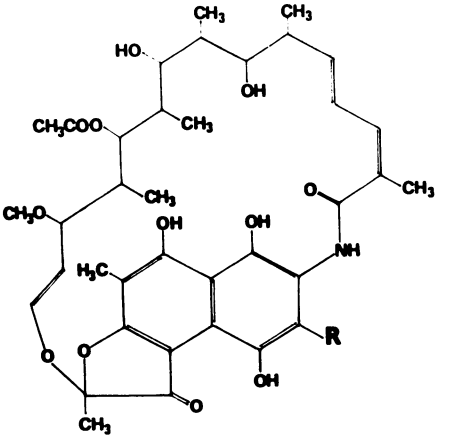
MATERIALS AND METHODS

Cell cultures. BHK-21 cells (clone 13) were grown as monolayers in 5-cm plastic petri dishes or 20-oz (ca. 600 ml) glass bottles in Eagles medium containing 10% (v/v) calf serum and 10% Tryptose phosphate broth.

Virus production and assay. Vaccinia virus (Evans vaccine strain) was grown in monolayers of BHK cells. Estimates of infectivity were made by plaque assay by using cell monolayers with a liquid overlay. Two days after infection, the monolayers were stained with Giemsa stain and the plaques were counted.

Ansamycin antibiotics and other inhibitors. Rifampin and rifamycin derivatives were supplied by G. Lancini, Lepetit Spa, Milan, Italy. Tolypomycin R was obtained from T. Kishi, Takeda Chemical Industries Ltd., Osaka, Japan, and supplies of streptovaricin were furnished by Calbiochem Ltd., London, and by Akio Nagata of the Toyo Jozo Co. Ltd., Tagata-Gun Shizuoka-Ken, Japan. Streptovaricin was supplied as a complex and was used without any attempt to separate the various components. Formulas, chemical nomenclature, and abbreviations of the rifamycin derivatives are shown in Fig. 1. Cycloheximide, puromycin, and cytosine arabinoside were purchased from the Sigma Chemical Co., St. Louis, Mo.; actinomycin was obtained from Merck, Sharp and Dohme, Ltd., Hoddesdon, Herts.

Electron microscopy. Confluent monolayers of BHK cells in 20-oz bottles were used in all experiments. Cells were removed from the glass by using a rubber policeman and resuspended in the final incubation medium. The cells were sedimented by centrifugation for 5 min at 300 × *g* and were then suspended in 2% glutaraldehyde. After 15 min of fixation at



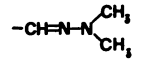
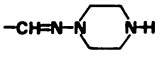
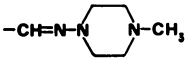
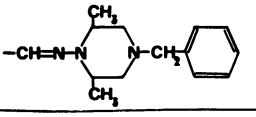
R	Designation used in text	No.
-H	Rifamycin SV	
-CHO	3-Formyl Rifamycin SV	1
-CH=NOH	39	39
	18	18
	N-Demethyl Rifampicin	30
	Rifampicin	31
	AF/ABDP	32

FIG. 1. *Ansamycin antibiotics, formulas, and nomenclature. The numbers in the right hand column are those used by Maggi et al. (9).*

room temperature, the cells were postfixed in 1% osmium tetroxide for a further 15 min, dehydrated in a graded series of alcohols, and embedded in Epon. Thin sections were cut on an LKB Ultrotome 1, stained with uranyl acetate and lead hydroxide, and examined in a Siemens Elmiskop la.

RESULTS

Effects of the ansamycin antibiotics on BHK cells Cell monolayers were treated with various concentrations of the test compounds for periods as long as 48 hr. At a concentration of 100 μg/ml, *N*-demethyl rifampin, compound 39, and the streptovaricin complex produced no obvious morphological changes in the monolayers. However, the remaining compounds (3-formyl rifamycin SV, rifamycin SV, compound 18, AF/ABDP, and tolypomycin R) produced severe toxic effects at this concentration, cells becoming rounded and detached from the plastic. In all cases the cell monolayers were completely destroyed within 48 hr. AF/ABDP and tolypomycin R were particularly toxic, 50 μg of these compounds per ml causing complete destruction of cell monolayers within 12 hr. At this concentration, cell monolayers treated with 3-formyl rifamycin and compound 18 showed only

minor morphological changes, some cell rounding, and an increase in the fragility of the monolayer being apparent. A striking change noted in thin sections of infected cells treated with rifampin (100 μg/ml for 17 hr postinfection; *see below*) and incubated for 90 min in the presence of 3-formyl rifamycin SV, AF/ABDP, and compound 18 at concentrations of 100 μg/ml was a gross swelling of the mitochondria, the cristae of which appeared as disorganized tags attached to the inner surface of the organelle (Fig. 2). This change was not seen in thin sections of rifampin-treated infected cells incubated with similar concentrations of the other ansamycin antibiotics.

Inhibition of virus plaque formation. No significant inhibition of plaque number and size was produced by *N*-demethyl rifampin, compound 39, and the streptovaricin complex at concentrations up to 100 μg/ml. AF/ABDP and tolypomycin R were tested at a concentration of 10 μg/ml; again no significant inhibition was detected. Plaque formation, however, was abolished by 3-formyl rifamycin SV and compound 18 at concentrations above 40 μg/ml (Table 1). The specificity of this effect was tested by examining the effect of these compounds on the plaque-forming ability of a rifampin-resistant variant of vaccinia (20). As with wild-type virus, 50 μg of the compounds per ml prevented plaque formation by the variant (Table 1).

Rifampin-induced maturation block and its reversal. As we wished to compare the antiviral activity of the test compounds with that of rifampin, the effect of these antibiotics on the reversibility of the rifampin-induced maturation block was examined by electron microscopy. This approach was chosen because it required only short periods of exposure of infected cells to test antibiotics, thus allowing the examination of toxic compounds, and because it allowed the detection of immature virus particles which cannot be unequivocally detected by any other procedure. It has been shown that the development of these particles after the removal of rifampin is not affected by inhibitors of DNA, RNA, and protein synthesis, (10–13). We, therefore, postulated that the replacement of rifampin by an ansamycin antibiotic with rifampin-like antiviral properties would result in the maintenance of the virus maturation block. Before

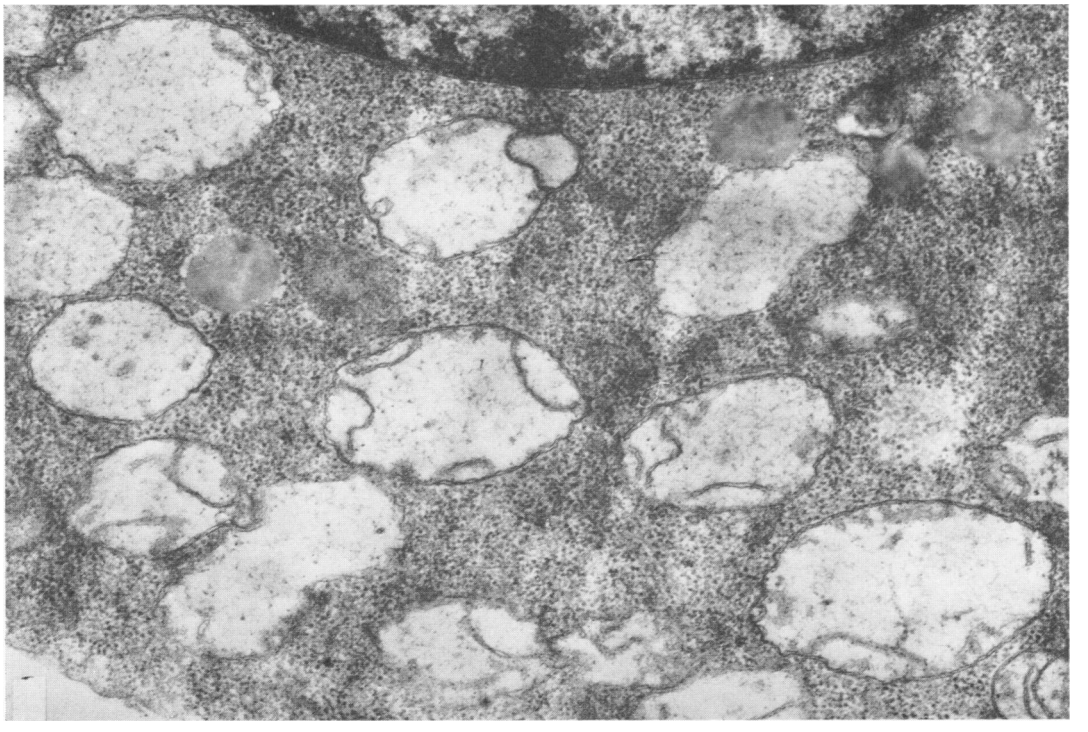


FIG. 2. *BHK-21/C13 cell after incubation in medium containing compound 18 (200 μg/ml) after infection with vaccinia virus in the presence of 100 μg of rifampin per ml. All mitochondria are grossly swollen with the cristae completely disorganized.* × *29,000.*

TABLE 1. *Inhibition of vaccinia virus plaque formation by 3-formyl rifamycin SV and compound 18*

Virus	Plaque no.[a]			
	Without inhibitor	3-Formyl rifamycin SV (50 μg/ml)	Compound 18 (50 μg/ml)	Rifampin (100 μg/ml)
Wild type	84	0	0	0
S (rifampin-resistant mutant)	81	0	0	27

[a] Plaque numbers represent the mean of four plates per assay.

describing the results of these studies, some features of the rifampin-induced maturation block and its reversal in the system used in this study are described, as our results differ in some details from the findings of other workers.

When cells infected with vaccinia virus are maintained in the presence of rifampin (100 μg/ml), characteristic electron-dense inclusions (Fig. 3) develop in the cytoplasm (11, 13). We have termed these R1 inclusions. They are much denser than other cytoplasmic organelles, are usually surrounded by a trilaminar membrane, and, in the system used in these experiments, often contain tubular structures (13). When rifampin is removed and replaced by normal medium, immature virus particles develop at the periphery of these inclusions (Fig. 4; references 11, 13). The development of these particles is preceded by the appearance of a regular array of spicules in the trilaminar membrane associated with the development of a characteristic curvature in the membrane. The regularity of this curvature, the uniformity in size of the complete immature virus particles, and the increased density of the spicule-covered membrane clearly distinguish immature and partially formed immature virus particles from all other cytoplasmic organelles and vesicles.

Kinetics of immature virus particle formation after removal of rifampin. The kinetics of immature virus formation after the removal of rifampin were examined in cells infected with vaccinia virus at an input multiplicity of 15 plaque-forming units per cell. After an adsorption period of 1 hr at room temperature, the cells were washed three times with medium and were then incubated in medium containing 100 μg of rifampin per ml for

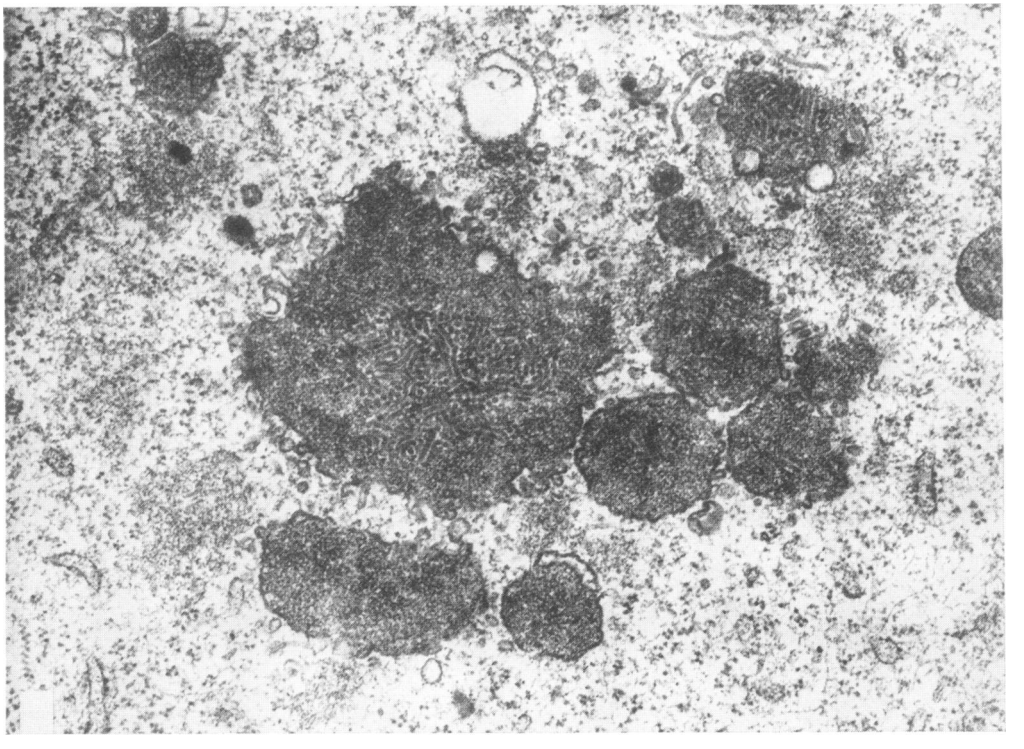


FIG. 3. *BHK-21/C13 cell 17 hr after infection with vaccinia virus in the presence of rifampin. Numerous R1 inclusions are present in the cytoplasm; some contain tubular structures.* × *32,000.*

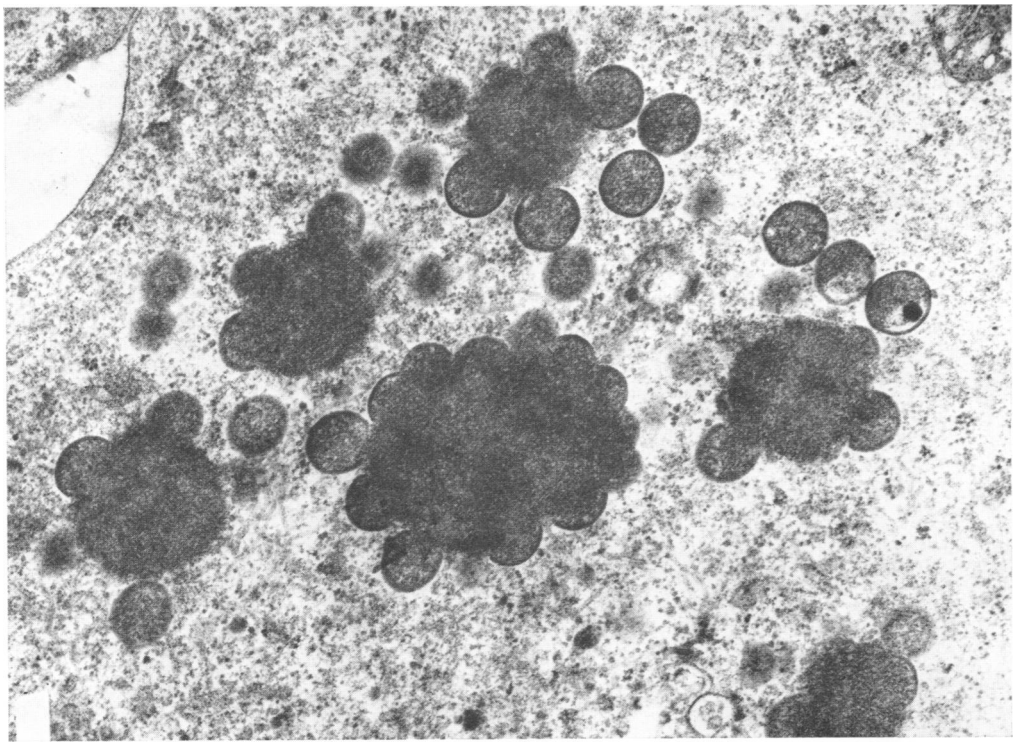


FIG. 4. *BHK-21/C13 cell after incubation in normal medium after removal of rifampin 17 hr postinfection. Immature particles are evident at the periphery of the original R1 inclusions and also free in the cytoplasm.* × *26,000.*

17 hr at 37 C. The medium was then poured off and immediately replaced with warm medium lacking rifampin. Samples were prepared for electron microscopy after fixation in situ with glutaraldehyde at room temperature for 15 min.

After incubation for 1 min in normal medium, no evidence of any development of immature virus particles was detected either at the periphery of the R1 inclusions or in the surrounding cytoplasm. In samples incubated for 2 min, typical curved immature particle membranes with associated spicules were seen at the periphery of the inclusions and in the surrounding cytoplasm (Fig. 5). Occasional short regions of markedly increased density were also observed at the periphery of some inclusions (Fig. 5); no curvature was apparent in these regions. Tubular structures were still present in some inclusions. Continued incubation for 5 and 10 min led to an increase in the proportion of inclusions with developing immature particles and to the appearance in the cytoplasmic matrix of complete immature particles apparently separated from the R1 inclusions. In samples incubated for 10 min, a small number of immature particles containing a dense nucleoid were noted. Further incubation resulted in the development of large numbers of immature particles, both with and without nucleoids, and morphologically complete virions. These findings are in general agreement with those of Grimley et al. (5); they did not, however, observe in their system the regions of increased density at the periphery of the R1 inclusions.

Effect of metabolic inhibitors on reversal of rifampin-induced virus maturation block. In HeLa cells and L cells infected with vaccinia virus, the development of immature virus particles from R1 inclusions is not prevented by inhibitors of DNA, RNA, and protein synthesis (10–12). We have confirmed these findings in our own system where, after the removal of rifampin, immature virus particles developed in the presence of 100 μg of cytosine arabinoside per ml, 10 μg of actinomycin D per ml, 300 μg of cycloheximide per ml, and 375 μg of puromycin per ml. Potassium cyanide and sodium azide (10^{-2} M) also failed to prevent the formation of immature particles after the removal of rifampin.

Effect of ansamycin antibiotics on reversal of rifampin-induced virus maturation block. Confluent monolayers of BHK cells were infected

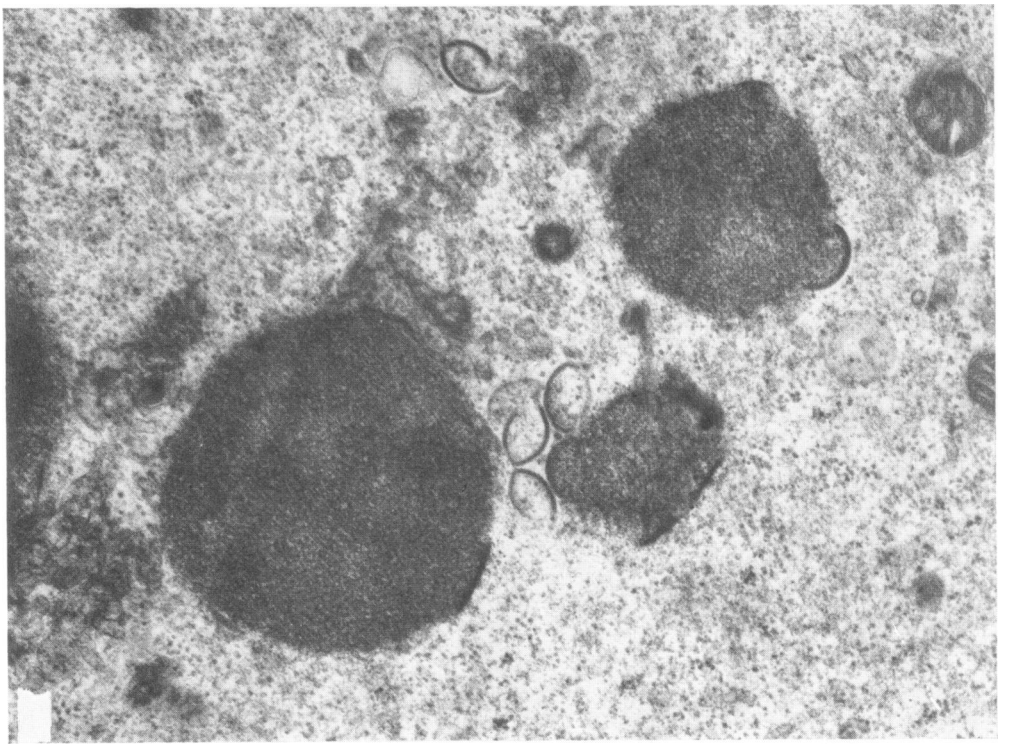


FIG. 5. *BHK-21/C13 cell after incubation for 2 min in normal medium after removal of rifampin 17 hr post-infection. Short, straight regions of markedly increased density are apparent at the periphery of some R1 inclusions. A curved region of immature particle membrane has developed at the edge of one inclusion. Immature particle membranes are seen free in the cytoplasm.* × *33,000.*

with vaccinia virus at an input multiplicity of 15 plaque-forming units per cell. After an adsorption period of 1 hr at room temperature, the cells were washed and incubated in medium containing 100 μg of rifampin per ml for 17 hr at 37 C. The medium was removed and replaced with fresh medium containing 100 μg of rifampin per ml as well as the appropriate concentration of the antibiotic under test. After incubation for 30 min, the medium was removed and replaced with medium containing the test antibiotic only. Incubation was then continued for at least 1 hr, at which time the cells were fixed and processed for electron microscopy.

All of the derivatives of 3-formyl rifamycin SV were capable of maintaining the rifampicin-induced maturation block (Table 2). Wide variations in the concentration required to maintain a complete block were found. Rifamycin SV and AF/ABDP were less effective than any of the other derivatives, producing only partial maintenance of the maturation block even at a concentration of 1,000 μg/ml.

Tolypomycin R and streptovaricin also proved capable of maintaining the maturation block produced by rifampin although high concentrations were required. In addition, tolypomycin R produced a marked increase in the granularity of the R1 inclusions (Fig. 6). This granularity was not apparent in control samples which had been

TABLE 2. *Effect of ansamycin antibiotics on the reversibility of the rifampin-induced virus maturation block*

Antibiotic	Minimal inhibitory concn[a]
Rifamycin SV	>1,000
3-Formyl rifamycin SV	200
Compound 39	500
Compound 18	100
N-demethyl rifampin	200
Rifampin	100
AF/ABDP	>1,000
Tolypomycin	300
Streptovaricin complex	1,000

[a] Compounds were tested at concentrations of 50, 100, 200, 500, and 1,000 μg/ml.

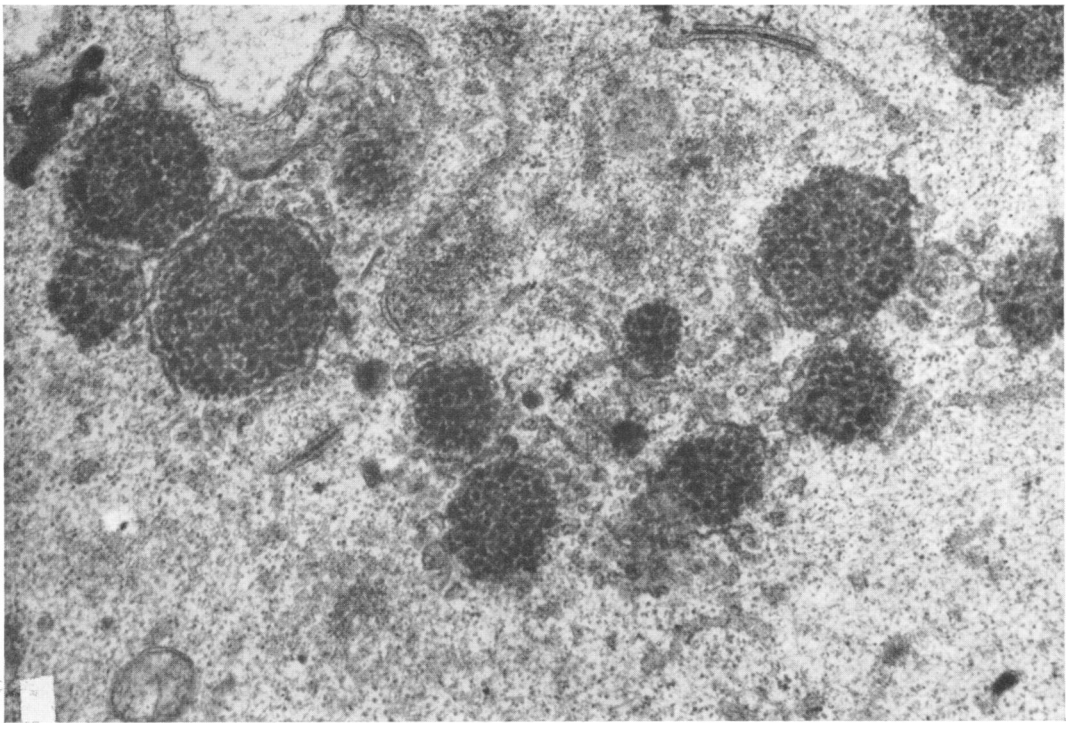


FIG. 6. *BHK-21/C13 cell after exposure to tolypomycin R (300 μg/ml) after removal of rifampin from vaccinia-infected cells. The original R1 inclusions have lost their uniform granular appearance and become mottled with densely stained areas.* × *29,000.*

maintained in rifampin throughout and which had been fixed and processed together with the sample incubated in tolypomycin.

Reversibility of maturation block maintained by antibiotics other than rifampin. The reversibility of the maturation block maintained by 3-formyl rifamycin SV and tolypomycin R was examined as follows. After substitution of the test compound for rifampin and subsequent incubation, the inhibitor under test was removed and fresh medium lacking inhibitor was added. Incubation was continued for 1 hr, and the cells were then fixed and examined. In experiments with both compounds, the rifampin-induced maturation block maintained by them was released after their removal, immature particles developing at the periphery of the R1 inclusions and in the cytoplasmic matrix. The granular appearance of the viroplasm produced by tolypomycin R persisted after removal of the drug, and complete immature particles contained material with a similar degree of granularity to that seen in the R1 inclusions. Immature particles containing granular material were also observed in cells treated with concentrations of tolypomycin R which only partially inhibited virus maturation (Fig. 7).

DISCUSSION

The experiments described here indicate that the block in vaccinia virus maturation induced by rifampin can be maintained by other ansamycin antibiotics. These observations contrast sharply with the failure of inhibitors of protein, RNA and DNA synthesis, and other general metabolic inhibitors to affect the development of immature particles from R1 inclusions after the removal of rifampin. We suggest that the maintenance of the virus maturation block by the ansamycin antibiotics we have tested indicates that these compounds exert a specific antiviral effect with the same molecular mechanism as that of rifampin. The reversibility of the maturation block maintained by 3-formyl rifamycin SV and tolypomycin is in accord with this hypothesis.

Comparison of the various rifamycin derivatives indicates that the nature of the side-chain attached to the 3-C position of the naphthohydroquinone moiety of the molecule exerts a profound effect on the potency of the rifampin-like antiviral effect. We have been unable to find any correlation between the chemical structure of this side-chain and antiviral activity. It is possible

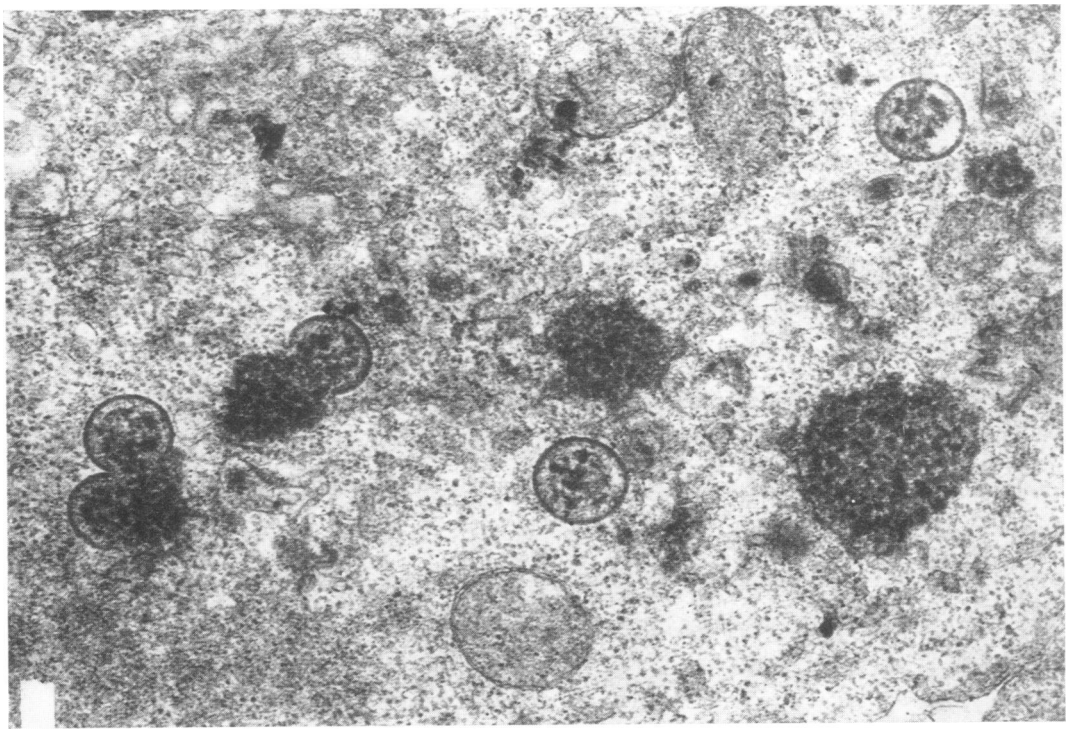


FIG. 7. *BHK-21/C13 cell after exposure to tolypomycin R (200 μg/ml) after removal of rifampin from vaccinia-infected cells. Both complete and partially formed immature particles can be seen enclosing densely stained heterogeneous material from the RI inclusions.* × *40,000.*

that these variations in antiviral activity reflect either differences in the ability of the compounds to enter the cells or to reach critical concentration at some specific location in the cell or differences in the interaction of the compounds with a viral component. However, it is clear that the specific antiviral activity of all these compounds resides in the rifamycin moiety. This conclusion differs from that of Thiry and Lancini (21) who reported that the antiviral activity of rifampin resides in the amino-methyl-piperazine side-chain of the molecule. Their studies, however, did not clearly show that the antiviral effect they observed was specific and unrelated to cytotoxic effects. We have already shown that in our system high concentrations of 1-amino 4-methyl piperazine display no specific antiviral activity [Follett and Pennington, Nature (London), *in press*].

It is clear that the inhibition of virus plaque formation by relatively low concentrations of 3-formyl rifamycin SV and compound 18 is not due solely to a rifampin-like effect, as both of these compounds inhibit plaque formation by rifampin-resistant vaccinia virus. Supporting this view is the observation that low concentrations of 3-formyl rifamycin SV and compound 18 have little inhibitory effect on virus maturation when substituted for rifampin late in infection, both compounds exerting a rifampin-like effect on virus maturation only at concentrations of 200 μg/ml and 100 μg/ml, respectively. Two possibilities which may explain these findings and which are under investigation are that 3-formyl rifamycin SV and compound 18 may exert a specific antiviral effect at a stage in virus development earlier than that of rifampin, possibly on the virus DNA-dependent RNA polymerase (*see below*), or alternatively that the inhibition of virus plaque formation follows the toxic effects of the compounds on BHK cells.

The streptovaricin complex tested in this study showed antiviral activity only at very high concentrations. It is possible that only a minor component of the complex possesses antiviral activity, a situation which would be similar to that found with cowpoxvirus, which, however, is inhibited by much smaller amounts of complex (14). Both streptovaricin and tolypomycin differ structurally from rifamycin SV in both the aliphatic chain and the chromophoric moiety. Tolypomycin R does not possess a side-chain attached to the 3-C position of the chromophore,

clearly indicating that such a side-chain is not necessary for rifampin-like antiviral activity. The significance of the granular appearance of the contents of R1 inclusions and immature virus particles occurring in cells incubated in the presence of tolypomycin is difficult to interpret. One possibility under investigation is that tolypomycin may bind to a viral component other than that involved in the rifampin-induced virus maturation block.

It has been shown recently that the compounds examined in this study exert an inhibitory effect on the virion-associated DNA-dependent RNA polymerase of vaccinia virus (Szilagyi and Pennington, *submitted for publication*). The results of this study showed that 3-formyl rifamycin SV and AF/ABDP at concentrations of 150 μg/ml and 100 μg/ml, respectively, produced a profound inhibition of enzymatic activity, this concentration of compound 18 inhibiting the enzyme partially. Raising the concentration of compound 18 to 500 μg/ml produced a complete inhibition of activity; a partial inhibitory effect was produced by this concentration of compound 39, tolypomycin R, and rifampin. There is no correlation between the concentration of antibiotic required to maintain the maturation block and that required to inhibit enzyme activity. Rifampin, tolypomycin R, and compound 18, for example, completely block virus maturation at concentrations less than those which produce a partial inhibition of enzyme activity, and, conversely, AF/ABDP produces a profound inhibition of enzyme activity at concentrations much lower than those required to block viral maturation. A further distinction between the ability of compounds to inhibit viral maturation and their ability to inhibit the virion RNA polymerase was indicated by the finding that the virion polymerase of mutants able to grow in the presence of 100 μg of rifampin per ml was inhibited by the compounds to the same degree as that of wild-type virus.

The observation that incubation of cells with many of the ansamycin antibiotics led to gross change in mitochondrial structure is of considerable interest in view of recent reports suggesting that rifamycins may inhibit eucaryote mitochondrial RNA synthesis (4, 17). Possible relationships between the cytotoxicity of these compounds and their effects on mitochondria are being further investigated.

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