

Interruption by Rifampin of an Early Stage in Vaccinia Virus Morphogenesis: Accumulation of Membranes Which Are Precursors of Virus Envelopes

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Assembly of vaccinia virus envelopes and immature vaccinia particles was interrupted in HeLa cells treated with rifampin (*rifampicin*). The primary action of rifampin on vaccinia morphogenesis appeared to occur during the stage of envelope formation. When envelopes and immature particles were already present, maturation could continue, even in the presence of rifampin. It was demonstrated that the trilaminar membranes of irregular contour which accumulate in the presence of rifampin are precursors of virus envelopes. When rifampin was removed under controlled conditions, synchronous transitions were observed as the precursor membranes rapidly converted into uniformly curved envelope units with a 10- to 12-nm coat on the convex surface. These experiments provided an opportunity to examine the sequence of some early events in vaccinia morphogenesis. Initially, nascent envelopes remained in clusters around dense viroplasm. Large numbers of single immature particles appeared within 10 min. Nucleation of immature particles was the first evidence of core differentiation and began within 5 to 10 min. Development of lateral bodies and modeling of the biconcave cores was observed within 30 min, and structurally mature virions were present by 2 hr after the removal of rifampin. High resolution autoradiography showed that viral deoxyribonucleic acid, which labeled with ^3H -thymidine during rifampin treatment, was incorporated by the mature vaccinia which formed after rifampin was removed. Concentration of the viral deoxyribonucleic acid in core material evidently occurred after envelope assembly, probably coincident with nucleoid formation. Cytoplasmic crystalloid bodies accumulated during rifampin treatment; they appeared morphologically identical to vaccinia nucleoids and were heavily labeled by ^3H -thymidine.

Rifampin, also known as *rifampicin* (20), is a semisynthetic antibiotic which can inhibit the production of infectious vaccinia virus (13, 35). Its antibacterial action is related to a direct effect on the function of ribonucleic acid (RNA) polymerase (10, 32, 38), but there has been no evidence for such an effect on the enzyme in purified vaccinia particles (6, 20, 24, 34). Data accumulated in several laboratories have shown that viral deoxyribonucleic acid (DNA) (3, 25, 34), species of viral messenger RNA (3, 19, 24), and a spectrum of both early and late vaccinia proteins (24, 25, 34) are made during rifampin treatment, even though the synthesis of late virus proteins declines with abnormal rapidity (3, 24).

Particles containing DNA cannot be isolated from cells which are infected in the presence of rifampin, but do form after the drug is removed (24, 25). Indeed, virus particles were isolated from HeLa cells after removal of rifampin despite a nearly total inhibition by cycloheximide of new protein synthesis (25). The latter finding suggested to us the possibility of a primary block in vaccinia assembly, and electron microscopy demonstrated that rifampin interrupted vaccinia virus assembly at a stage in the formation of virus envelopes (25). Some similar morphological observations were recently reported by Nagayama et al. (27) studying infected L cells, but in that system treatment with streptovitacin appeared to

prevent maturation of particles after removal of rifampin. Although a rise in the activity of RNA polymerase (19, 27) and other late enzyme components of vaccinia particles (27) cannot be detected in cells treated with rifampin, it now seems possible that these may be secondary phenomena related to the virus assembly block (15a).

The controlled removal of rifampin provides a unique experimental conditions in which the early stages of assembly and maturation of poxvirus elementary particles are relatively synchronous and can be examined sequentially. The present report details the morphological effects of rifampin on vaccinia virus development in HeLa cells and also describes transitional steps of envelope and particle assembly from the envelope precursor membranes which form during rifampin treatment. The fate of viral DNA which replicates during rifampin treatment was followed by high resolution autoradiography.

MATERIALS AND METHODS

Virus and cells. The strain WR of vaccinia virus was propagated in HeLa cells, and virus titers were measured by a plaque assay on HeLa cell or chick embryo cell monolayers (24, 25). The HeLa cells at a concentration of 2×10^6 to 5×10^6 /ml were maintained at 37°C in suspension culture of Eagle's medium containing 5% horse serum. HeLa cells (4×10^6 /ml) were infected with purified virus (24, 25) at a multiplicity of 30 plaque-forming units (PFU) per cell. After 30 min, the cells were washed three times with fresh Eagle's medium and suspended at a concentration of 4×10^6 /ml.

Materials. Rifampin was obtained from Dow Chemical Co., Zionsville, Ind., and was added to cultures as previously described (25). The ^3H -thymidine (14 Ci/mmol) was obtained from New England Nuclear Corp., Boston, Mass.

Electron microscopy. Portions of the cell suspensions were centrifuged at $800 \times g$ at 4°C for 1 min. The supernatant fluid was immediately replaced by fixative solution (4°C), and the cohesive cell pellets were gently dislodged from the sides of the centrifuge tubes to ensure even fixative penetration. Several fixative solutions were employed, including glutaraldehyde from 2 to 6% in 0.1 M Sorenson's buffer at pH 7.4 (5), 3% formaldehyde prepared directly in Sorenson's buffer by warming a suspension of trioxymethylene powder (Fisher Scientific Co., Fairlawn, N.J.) to 60°C, 1% OsO₄ in phosphate buffer (21), or 0.6% KMnO₄ buffered with acetate-Veronal (18). The aldehyde-fixed cell pellets were post-treated with 1% OsO₄, dehydrated in ethanol solutions, and embedded in Luft's Epon formula as previously reported (12, 25). Some aldehyde-fixed pellets were transferred to 5% uranyl acetate in methanol after the ethanol dehydration to enhance membrane contrast, and some OsO₄-

fixed pellets were treated with 0.1 N sodium acetate and 0.5% uranyl acetate (36). Ultrathin sections were obtained with an automatic ultratome and diamond knives. They were stained with 5% uranyl acetate in methanol or with saturated lead citrate (25) before examination in a Perkin-Elmer, Hitachi HU 11-E microscope.

Autoradiography. HeLa cells infected in the presence of rifampin were pulsed from 2 to 2.5 hr after infection with 10 μCi of ^3H -thymidine per ml. Cells were then sedimented, washed, and suspended in medium containing rifampin and excess unlabeled thymidine (10^{-6} M). Cell pellets for autoradiography were fixed with 3% formaldehyde and were prepared as above for electron microscopy. No more than 5% of the radioactivity is extracted during this type of procedure, and artefactual binding of ^3H -thymidine is negligible (37). Based on methods of Caro (4), Ilford L-4 emulsion (Nuclear Research, Ilford Ltd., Ilford, Essex, England) was melted and mixed in proportions of 2:3 with distilled water. Ultrathin sections for electron microscopy and 0.5- to 1- μm sections for light microscopy were mounted on Formvar-coated titanium grids or glass slides and coated with loops of gelled emulsion (12). Uniformity of undeveloped and exposed-developed control films was tested within each group of experiments (12). Background grains with a Kodak safelight filter (w ratten series no. 2) and up to 6 weeks of exposure were negligible (less than one grain per Fullam Effa-Ti no. 2202 grid slit). Growth of the exposed silver grains was controlled by use of Microdiol-X or Kodak D-19 developers and varying the duration of development (16). After development, sections were stained for electron microscopy by submersion in nearly saturated lead citrate for up to 15 min, or they were examined without staining. Sections for light microscopy were stained through the emulsion with 0.1% Toluidine Blue and rinsed briefly in dilute acid alcohol.

For grain counts, the center of a circle around each grain was considered the source of radioactivity (29). Appropriate regions were outlined on glossy micrographs (Kodak F-5 paper) which were magnified by 2.5 times the original negative plates. The magnified areas were measured by cutting out and weighing the outlined portions and converting by a predetermined factor (1 g = 55.6 cm²). The original area in μm^2 could then be calculated.

Detection of DNA-containing virus particles. Cells treated with rifampin or untreated controls were incubated with ^3H -thymidine (1.0 $\mu\text{Ci}/\text{ml}$) from 2 to 4 hr after infection. The pulse was terminated by sedimenting the cells and resuspending them in fresh medium containing unlabeled thymidine (10^{-6} M) with or without rifampin. At intervals thereafter, 10-ml samples of cells were removed and radioactively labeled virus was purified by sucrose gradient sedimentation by procedures described previously (25).

RESULTS

Effect of rifampin on vaccinia virus morphogenesis. In the continuous presence of rifampin (100 $\mu\text{g}/\text{ml}$) which was added 10 min prior to

infection (25), foci of viroplasm became evident within 2.5 hr. Ultrastructurally, these were indistinguishable from the normal viroplasmic foci previously described in several poxvirus infections (15, 22, 30). The viroplasm coalesced into large masses of granular and amorphous material which localized in the cytocentrum and measured up to 7 μm in greatest dimension. Except for short profiles of granular endoplasmic reticulum or zipper-like arrays of polyribosomes (9, 15), cellular organelles were generally excluded from the viroplasm (Fig. 1).

At 4 hr after infection, the first differences from normal vaccinia development were noted in rifampin-treated cells. Discrete regions of the viroplasm appeared to condense and increase in electron-scattering power (Fig. 2). These regions, described as domains (25), were characteristically associated with irregular profiles of "unit" membrane (Fig. 2, 3a). The membrane profiles formed a boundary between the condensed viroplasm of domains and adjacent viroplasm or cytoplasm of lesser electron density. They displayed a trilaminar substructure after primary fixation with aldehydes, OsO_4 , or KMnO_4 , and the total width averaged approximately 8 nm (Fig. 3b).

When rifampin was added before infection, the

numbers of membrane profiles associated with individual domain cross-sections and the total number of membrane-limited domains increased progressively for 8 hr (Fig. 4). Continuous membrane profiles of up to 1.5 μm in length were measured. Eventually, domains were almost completely encompassed by unit membranes, and the membrane-limited domains averaged approximately 0.6 μm in breadth. The numbers of membrane-limited domains increased only slightly from 8 to 12 hr after infection (Fig. 4), a time when the rate of viral protein synthesis decreases significantly (3, 24). After 24 hr in the presence of rifampin, many viral membranes were detached from the domain regions, and spherical membrane micelles or laminated membrane stacks collected in the cytoplasm. The viroplasm contained large masses of fibrillar material (Fig. 5).

Normal developmental forms of vaccinia virus, including envelopes and immature particles, were only rarely identified in cells treated with 100 μg of rifampin per ml from the beginning of infection. They did not appear until after 6 hr, and constituted only a minute fraction of the number of virus particles seen at comparable times in control infections (Fig. 4).

Membrane-limited domains formed when

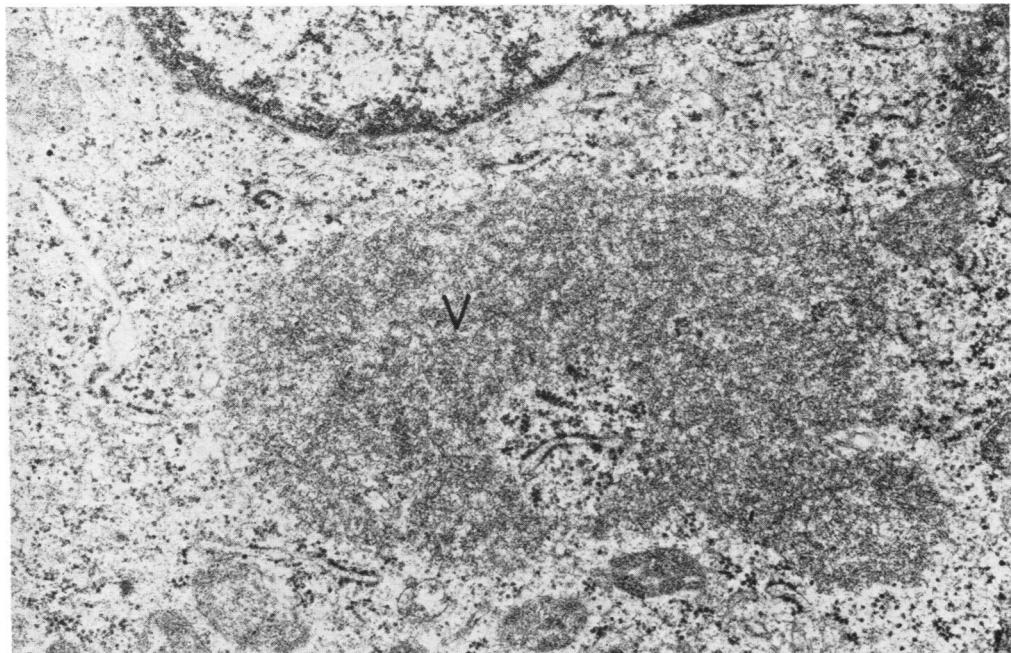


FIG. 1. HeLa cell treated with rifampin and infected with vaccinia virus for 3 hr. Finely granular and amorphous viroplasm (V) surrounds a small portion of cytoplasm including ribosome arrays and a profile of granular endoplasmic reticulum. $\times 24,400$.

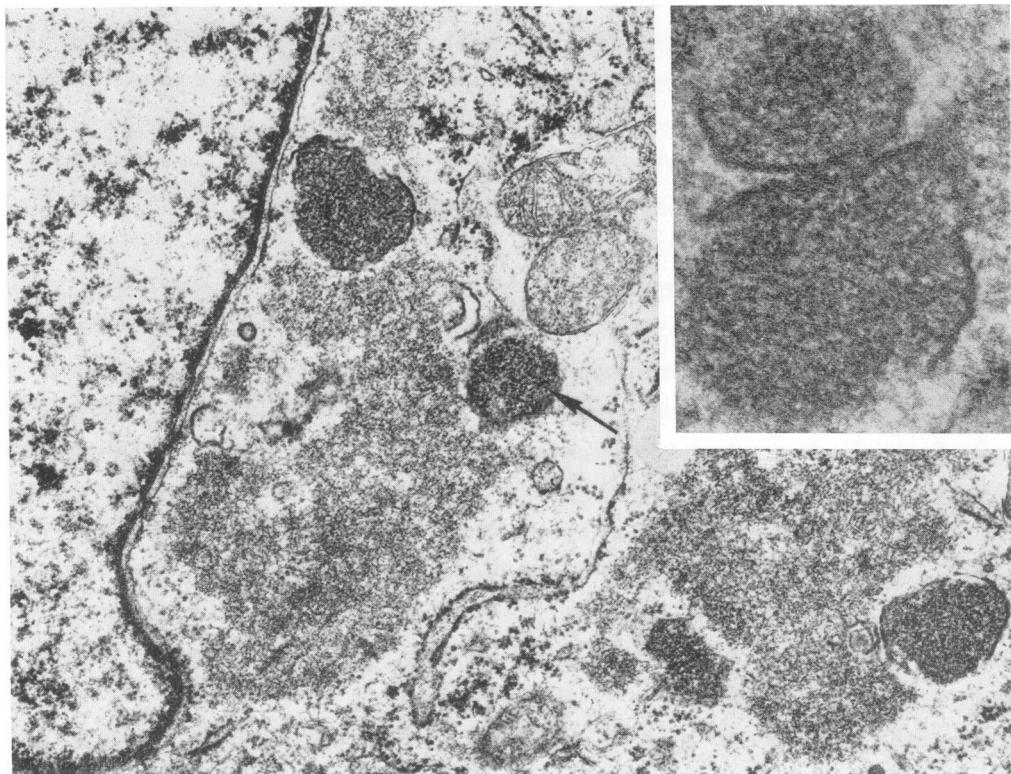


FIG. 2. *HeLa* cell treated with rifampin and infected with vaccinia virus for 4 hr. Differentiated regions of viroplasm (domains) show increased electron density (arrow). $\times 24,500$. Domains (insert) are partially delimited by trilaminar membranes. $\times 80,000$.

rifampin was added to HeLa cells at times up to 10 hr after the beginning of a vaccinia infection. They could be identified within 30 min after addition of the antibiotic (Table 1). In general, the envelope precursor membranes and domains appeared to arise without relation to preexisting immature envelope crescents or particles (Fig. 6a). Rarely, preexisting envelope crescents were demonstrably contiguous with uncoated membrane "fringes" which evidently formed subsequent to the addition of rifampin (Fig. 6b). In other cases, there was a sharing of domain viroplasm by envelope crescents and precursor membranes with no apparent interconnection (Fig. 6c).

Maturation of vaccinia particles evidently continued when rifampin was added at 4 to 6 hr after the start of infection. An increased number of particles labeled with ^3H -thymidine could be isolated from the cells for at least 2 hr (Fig. 7), and the percentage of immature particles in ultrathin sections declined (Table 1).

Assembly of virus envelopes and immature particles after removal of rifampin. The reversability of

the rifampin effect on vaccinia infection (25, 34) and the formation of complete virions has been reported (25). New morphological observations demonstrated that the membranes which arise in association with domains during rifampin treatment are directly converted into virus envelopes. Controlled removal of rifampin from infected cells was critical for the evaluation of the early changes. Cells were washed in ice-chilled medium without rifampin (25) and were resuspended in fresh prewarmed medium (37°C). Samples were prepared for electron microscopy at exact intervals by pouring cell suspensions over frozen-crushed medium in large centrifuge tubes and sedimenting pellets for cold fixation.

No significant membrane changes occurred during the cold wash. During the first minute of rewarming, focal accretions of coat material were detected on the exterior surface of membranes limiting the domain regions (Fig. 8a). Coated portions of membrane became slightly curved and more regular in appearance (Figs. 8a, 8b). Within 2 min after rewarming, distinct regions of membrane coating could be detected at the periphery

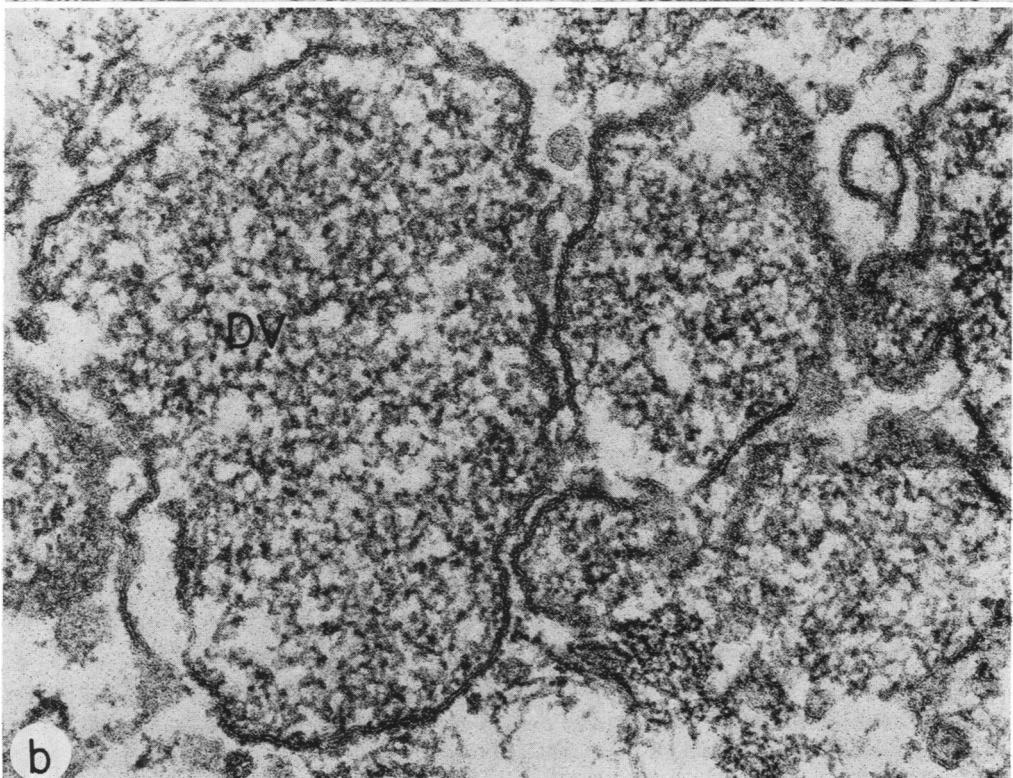
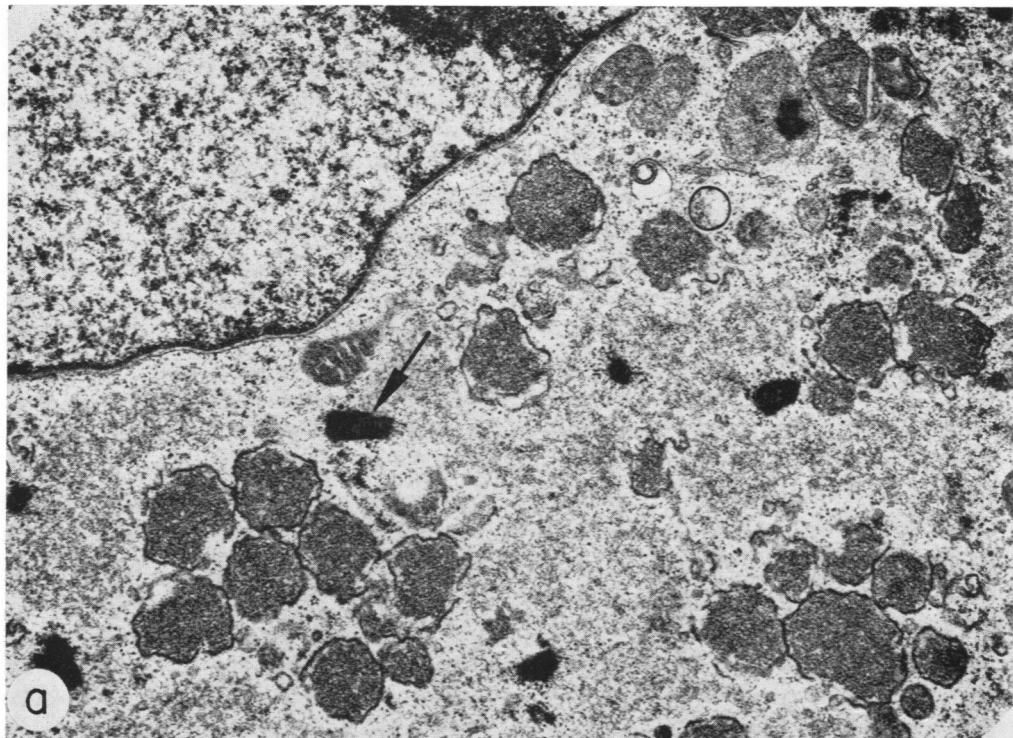


FIG. 3. Membrane-limited domains in HeLa cell treated with rifampin and infected with vaccinia virus for 8 hr. a, Low magnification illustrating groups of membrane-limited domains in cytocentrum. Dense bodies (arrow) are DNA-rich crystalloids (see Fig. 12). $\times 17,200$. b, Enhanced contrast of the trilaminar membranes around domains was produced by direct OsO_4 fixation followed by uranyl acetate. This resulted in a coarser aggregation of domain viroplasm (DV). $\times 80,000$.

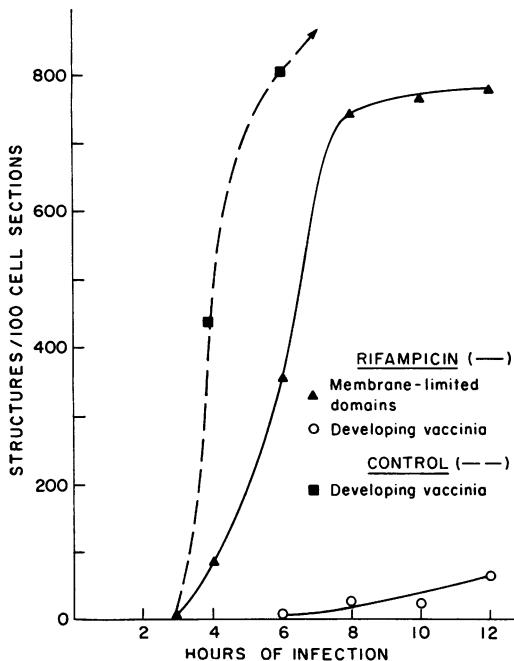


FIG. 4. Sequential counts of membrane-limited domains and total developing vaccinia particles (types I-VI) in ultrathin sections of HeLa cells infected with vaccinia virus in the presence of rifampin. The control was a parallel culture infected without rifampin.

of nearly all membrane-limited domains (Fig. 8c), and there were obvious transitions between irregular precursor membranes and typical virus envelopes (Fig. 8c). By 5 min, the transitional phase of membrane coating was nearly concluded. More than one curved envelope unit apparently could be fashioned out of a single sheet of precursor membrane, and the envelopes often remained in contiguous clusters around the original membrane-limited domains (Fig. 9).

Since some previous studies of poxvirus morphogenesis had suggested that individual elementary bodies might arise by detachment from complexes of envelopes associated with dense viroplasm (1, 2, 22), similar to the clusters observed after rifampin treatment (Fig. 9), the present experiments appeared to offer a unique condition for examining the dynamics of particle formation. For this reason, structures per 100 cell sections were enumerated at various times after the removal of rifampin. Owing to the relative dimensions of the ultrathin sections (80 to 100 nm) and of the spherical immature vaccinia (300 to 350 nm), slices of incomplete particles which are shaped as open cupules (1) can appear either as crescentic or circular profiles, depending upon the plane of

section. In the maturational scheme outlined by deHarven and Yohn (9), crescentic profiles were classified as type I particles, and circular profiles were classified as type II particles. Enumeration of these forms, although arbitrary, can provide a useful index of the overall development toward complete elementary bodies (7). In Fig. 10, counts of type I and type II particles are compared to the relative numbers of clusters containing four or more crescentic envelope profiles. Individual envelope units (type I or type II particles) began to appear within 2 min after the removal of rifampin, but in absolute numbers per 100 sections they were less numerous than envelopes in clusters until after 3 min. After 5 min, the numbers of envelopes in clusters declined, while numbers of individual immature particles continued to increase. Numbers of membrane-limited domains showed a steep decline within 2 min. The overall stoichiometry was consistent with the formation of multiple particles from each membrane-limited domain and suggested that the envelopes in clusters gradually dissociated. From 10 to 30 min after the removal of rifampin, there was no remarkable shift in the numbers of type II particles observed.

Formation of viral nucleoids and later maturation of vaccinia particles. Dense viroplasm of the domain regions was contiguous with core matrices of particles which appeared to be separating from clusters (Fig. 9). Modification of the core evidently occurred after this separation. Differentiation of nucleoids within a small proportion of immature vaccinia first occurred between 5 and 10 min after the removal of rifampin. These were defined as type III particles (9). Nucleoids were not observed in hundreds of immature forms examined at 5 min or earlier. The nucleoids were usually located at the periphery of circular particle profiles and were never observed within an envelope cavity when the circumference of a profile was less than 90% completed. Rare images after removal of rifampin or in untreated cells suggested that condensation of the nucleoid material sometimes occurred just before the vaccinia envelope completed, and this has been confirmed by more extensive investigation (P. M. Grimley, *unpublished observations*). A similar observation was reported in a study of ectromelia infection by Leduc and Bernhard (17). The proportion of immature particle profiles showing nucleoids remained at about 10% from 10 to 30 min after withdrawal of rifampin and was slightly lower at 1 hr. As shown by Morgan et al. (23), these proportions are not absolute, but should reflect the number of nucleoids in a ratio roughly 1/3 to 1/5.

Vaccinia particles with lateral bodies (type IV

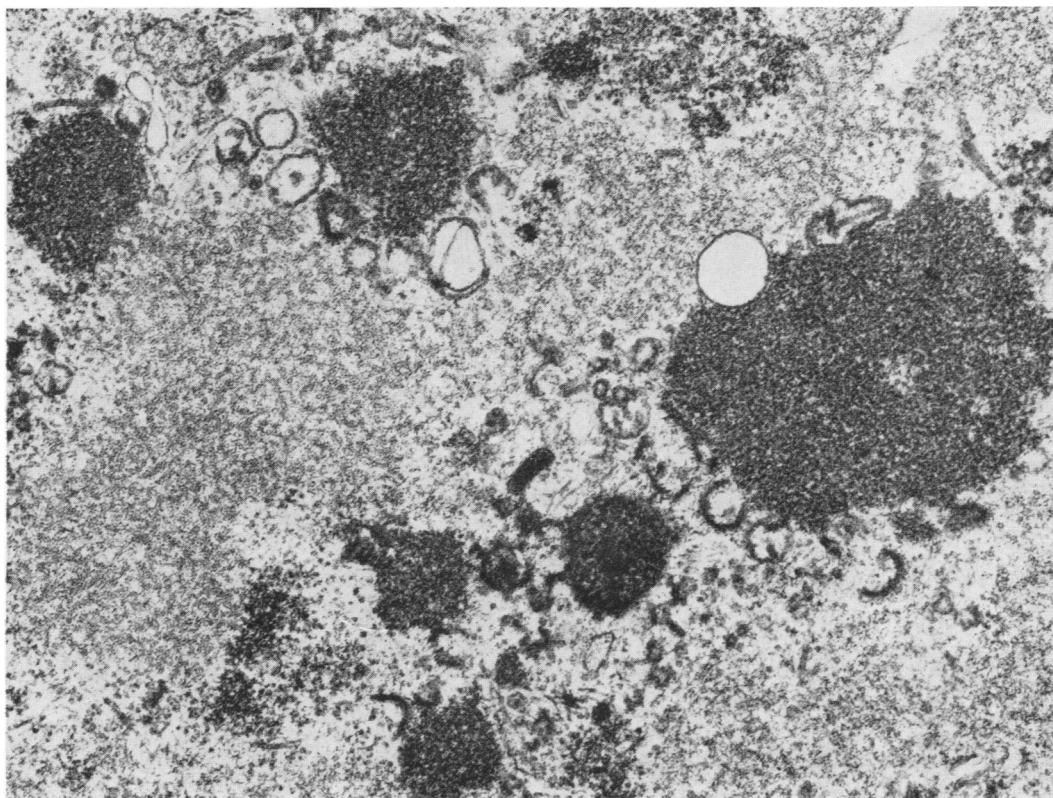


FIG. 5. HeLa cell treated with rifampin and infected with vaccinia virus for 22 hr. Membranes no longer delimit domain regions, and some form spherical structures. Portions of viroplasm display a fibrillar pattern. $\times 30,500$.

TABLE 1. Differential counts of vaccinia virus particles identified after addition of rifampin^a

Time after infection hr	No. of MLD	Total vaccinia particles	Immature	Mature
6	0	1,400	47	53
7	62	1,250	45	55
9	110	1,140	14	86

^a Rifampin was added at 6 hr after infection. Membrane-limited domains (MLD), total vaccinia particles, immature particles (types I-III), and mature particles were counted in thin sections of 100 cells. A decline in the total number of vaccinia was presumably due to continuous release of virions (8). In parallel controls without rifampin, the number of vaccinia nearly doubled from 6 to 9 hr.

particles) were observed as early as 30 min after the removal of rifampin but not before 20 min. Clusters of more mature vaccinia (types V or VI particles) were apparent by 2 hr, a time when

increased titers of infectious virus could first be detected by plaque assay (25).

Autoradiographic localization of viral DNA. Light microscopic localization of ^{3}H -thymidine in the cytoplasm of vaccinia-infected cells treated with rifampin was reported by Subak-Sharpe et al. (34). The electron density of the matrix material composing membrane-limited domains suggested that it might be preferentially enriched in viral DNA. For our high-resolution experiments, cells were pulsed with ^{3}H -thymidine from 2 to 2.5 hr after infection. This represented a period of maximal ^{3}H -thymidine incorporation in the presence of rifampin (25). Figure 11a shows that developed silver grains were discretely localized over viroplasmic foci at 2.5 and 4 hr. This was also clear in 1- μm epoxy sections examined by light microscopy. In cells examined at 8 hr, grains were not concentrated over those regions of viroplasm which could be distinguished as membrane-limited domains. Groups of membrane-limited domains were often unlabeled, whereas the adjacent viroplasm was heavily labeled (Fig. 11b). At

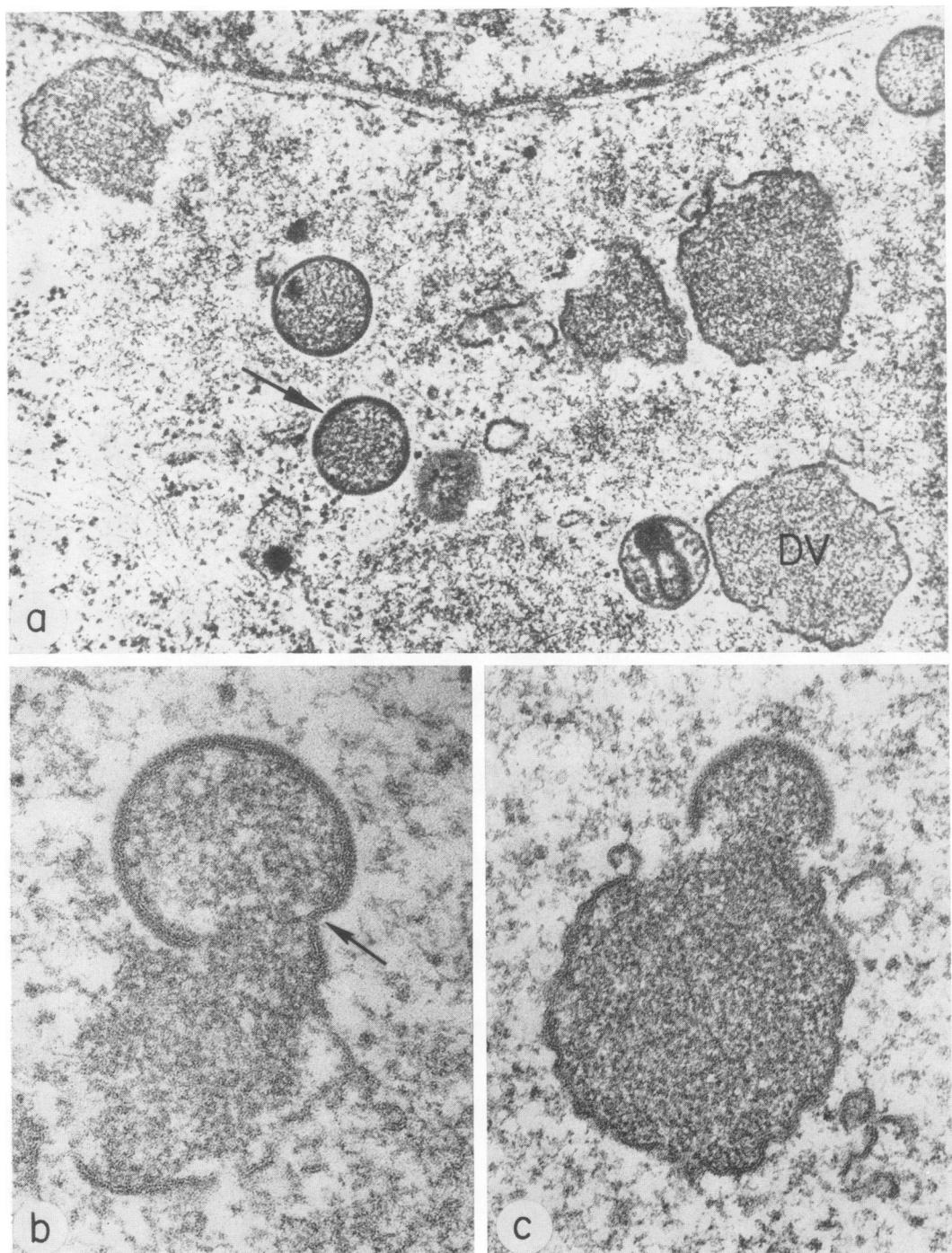


FIG. 6. Fields in HeLa cells infected with vaccinia virus for 4 hr, treated with rifampin from 4 to 6 hr, and then fixed. *a*, Membrane-limited domains of viroplasm (DV) in proximity to immature and maturing virus particles (arrow). $\times 33,600$. *b*, Continuity (arrow) of uncoated trilaminar membrane with an immature envelope unit and sharing of viroplasm. $\times 95,200$. *c*, Sharing of viroplasm without evidence of continuity between coated and uncoated membranes. $\times 75,000$.

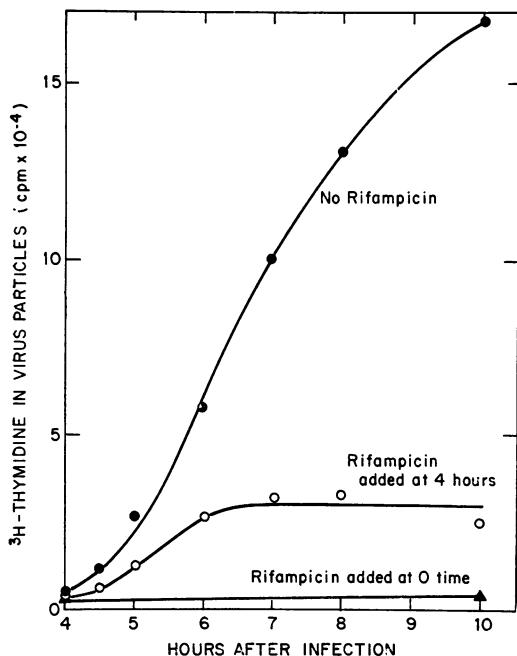


FIG. 7. Formation of ^{3}H -thymidine-labeled virus particles after the addition of rifampin. HeLa cells were incubated with ^{3}H -thymidine from 2 to 4 hr after vaccinia infection. One portion of cells was treated with rifampin throughout the infection, another received the drug at 4 hr after infection, and a third portion was not treated with rifampin.

8 hr after infection, rifampin was removed and the fate of viral DNA was followed. Within 30 min, developed grains could be observed over immature particles which contained nucleoids (Fig. 11c). By 4 hr after removal of rifampin, groups of virions were specifically labeled with developed grains (Fig. 11d).

The qualitative observations were supported by comparative grain counts. In repeated experiments, the number of grains per unit area of viroplasm appeared slightly but consistently lower than the number of grains per unit area of total viroplasm (Table 2). Comparison of grains counted over 400 membrane-limited domains which were present at 8 hr and over an equal number of virions which were examined at 4 hr after removal of rifampin gave the following results: 20 grains per 100 domains and 33 grains per 100 virions. This difference appeared to be of significance, since the area of a typical membrane-limited domain is much larger than a single virion (Fig. 6a), and each domain can apparently give rise to several virions (Fig. 9, 10).

Large crystalloid bodies of high electron density were a prominent feature in the cytoplasm of

infected cells treated for 6 hr or longer with rifampin (Fig. 12). They were uniformly labeled by the ^{3}H -thymidine (Fig. 12c). The density and substructure of crystalloid bodies resembled that of vaccinia nucleoids (Fig. 12b). Periodic fibrils, measuring 4 to 6 nm in diameter, extended along the length of exposed crystalloid planes, with an interfibrillar distance of 5 to 8 nm. Similar crystalloid bodies have been identified during the natural course of Shope fibroma infections (31) and were observed in mouse L cells infected with vaccinia virus (8, 27). In present experiments, they were not usually observed in hundreds of sections of HeLa cells infected with vaccinia virus for up to 22 hr, except after rifampin treatment.

DISCUSSION

It remains to be determined whether rifampin directly blocks bonding of a coat material to vaccinia precursor membranes or whether it blocks some antecedent step in biogenesis. The possibility of a block in assembly of some key molecule remains to be explored (35). After removal of rifampin, envelope coating proceeds rapidly despite the presence of potent metabolic inhibitors (P. M. Grimley and B. Moss, Fed. Proc. p. 309, 1970), and progeny particles have been demonstrated repeatedly even in the absence of significant new protein synthesis in Hela cells (25). Although suggested by others (27), there has been no direct evidence that rifampin exerts its antiviral action by inhibiting the synthesis of specific protein. Katz and Moss (15b) (*in press*) showed that cleavage of a structural protein precursor fails to occur in the presence of rifampin, but this is interpreted as an effect which is secondary to the block in envelope assembly (15a, 15b).

Initial ultrastructural observations showed that the action of rifampin on vaccinia assembly occurs at a stage prior to the complete formation of virus envelopes (25). Present experiments established that trilaminar membranes which accumulate in the presence of rifampin are direct precursors of vaccinia envelopes. A two-step process of poxvirus envelope assembly thus can occur under specific experimental conditions. In previous studies, poxvirus envelope formation has always appeared to be a concerted process in which the virus envelopes displayed an outer coat and curved geometry at the earliest times of identification. If stepwise assembly of vaccinia envelopes does occur naturally, it may be too fleeting for morphological detection or occur in units below the limits of practical resolution. Treatment of L cells with actinomycin D by Dales and Mossbach (7) resulted in the aberrant formation of small regions or "micelles" of virus membrane which

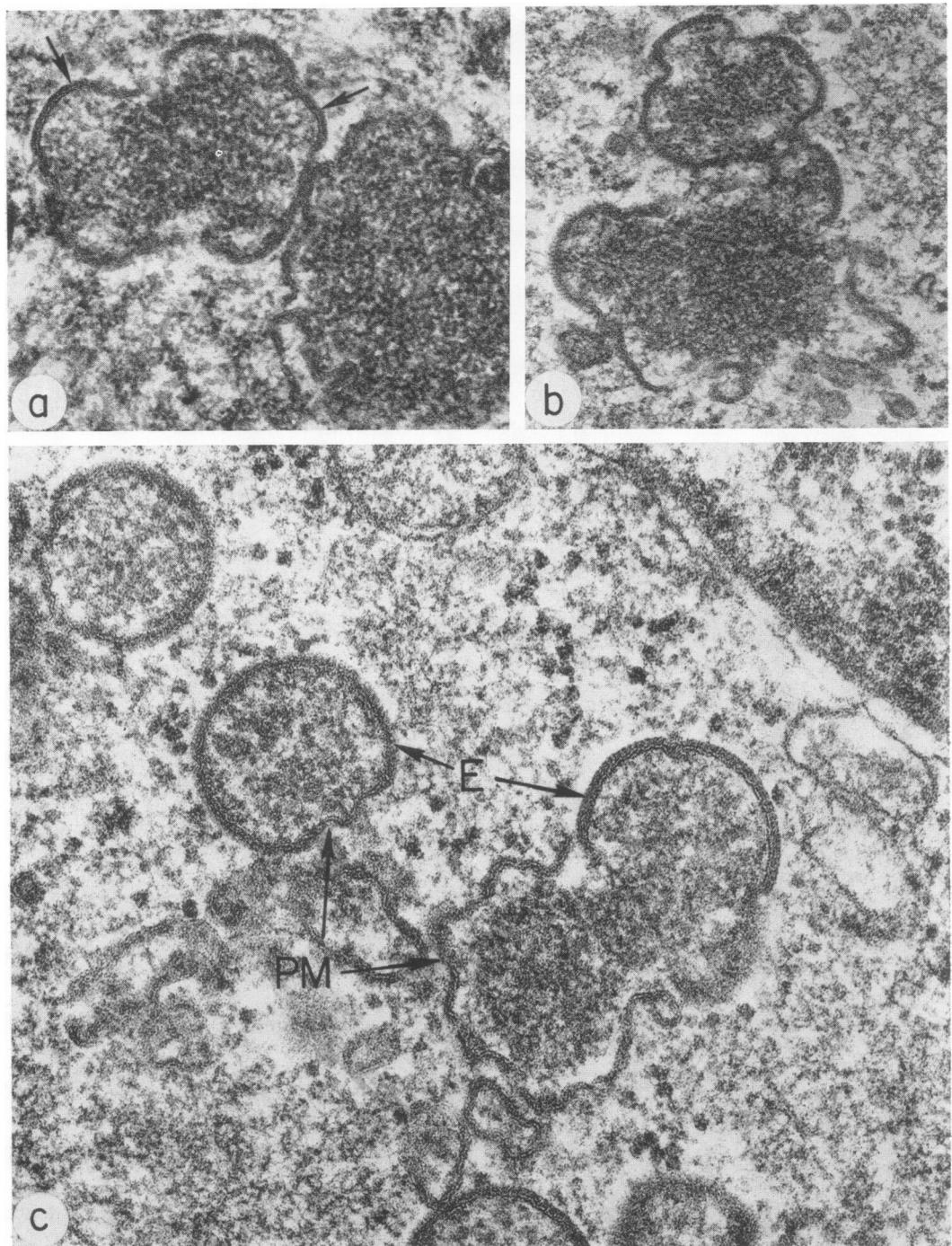


FIG. 8. *Transitions from precursor membranes to vaccinia virus envelopes. HeLa cells were infected for 8 hr in the presence of rifampin, and then the drug was removed under controlled conditions. a, Partial coating (arrows) of domain membrane within 1 min after removal of rifampin. $\times 65,000$. b, Progressive coating and curvature of membranes at 3 min after removal of rifampin. $\times 65,000$. c, Continuity of coated envelopes (E) with uncoated precursor membrane (PM) at 3 min after removal of rifampin. $\times 97,500$.*

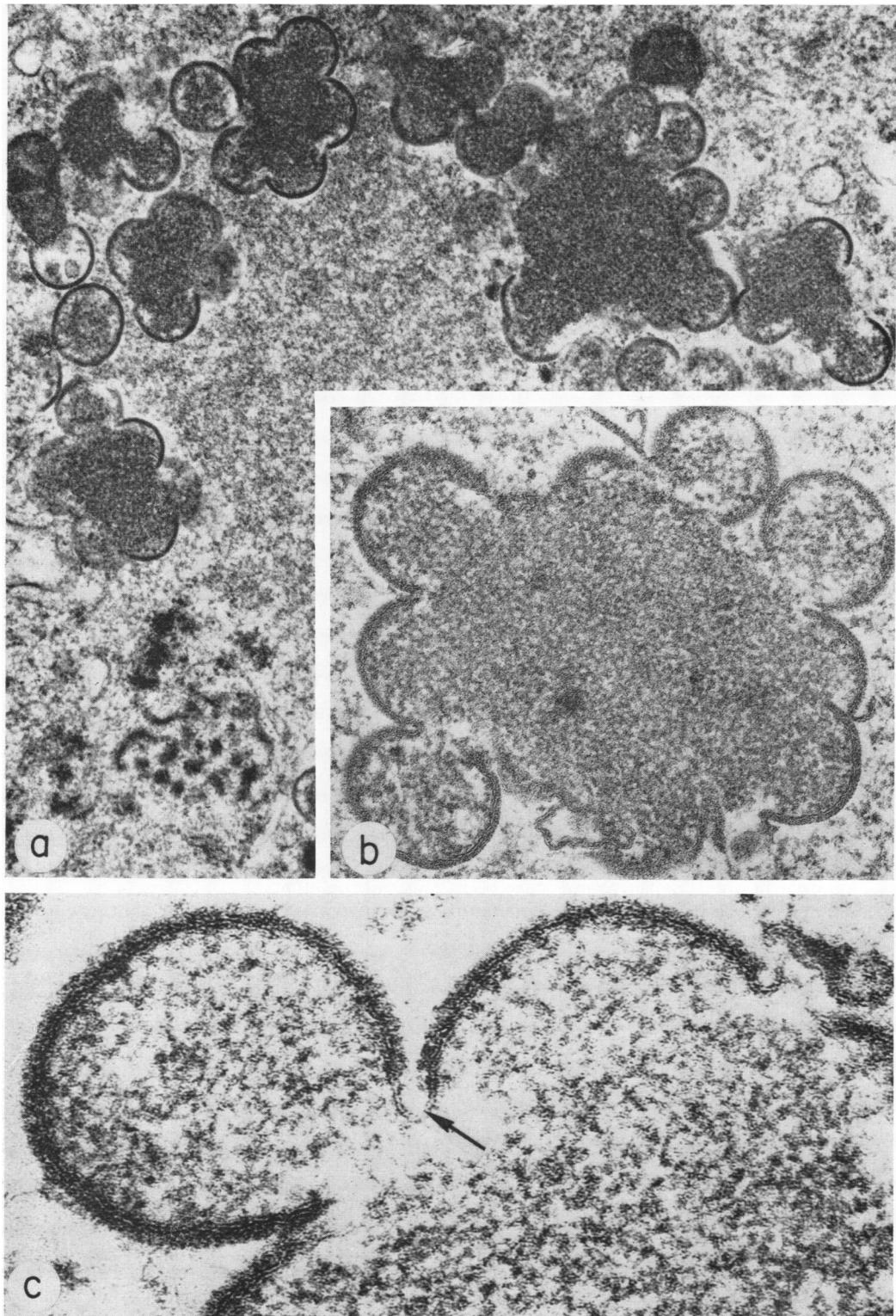


FIG. 9. Clusters of envelope units replace precursor membranes by 10 min after removal of rifampin (same conditions as Fig. 8). *a*, Low magnification of envelope clusters which persist up to 4 hr. $\times 31,800$. *b*, Image suggesting continuous modeling and separation of nearly complete envelopes at 10 min. $\times 67,500$. *c*, High resolution of nascent envelopes demonstrating continuous U-shaped linkage of trilaminar membranes through an uncoated segment (arrow). $\times 156,000$.

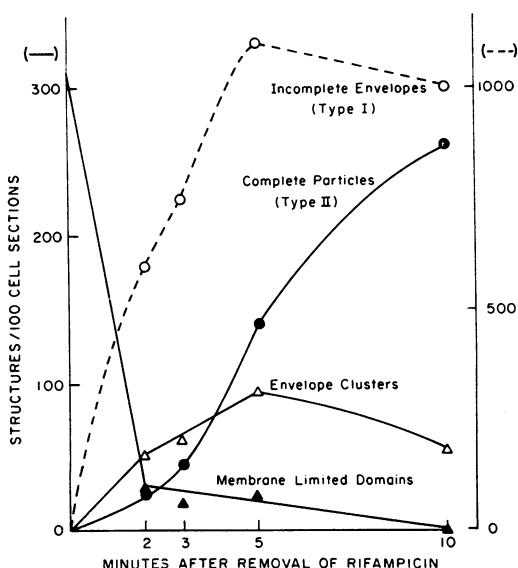


FIG. 10. Counts of membrane-limited domains, clusters of envelopes, incomplete envelopes (type I particles), and complete vaccinia (type II particles) in thin sections of cells after controlled removal of rifampin. Cells were treated with rifampin during 0 to 8 hr of infection.

lacked an exterior coat, but this effect was not shown to be reversible.

An association of developing vaccinia envelopes with dense regions of the viroplasm, similar to the domains now described, has often been noted in the course of poxvirus infections (1, 2, 8, 15, 17, 22, 30). In general, incomplete poxvirus envelopes which appear in profile as "cresecnts" have been situated at the periphery of the dense viroplasm with convexity outwards. Under the conditions of rifampin treatment, there is a consistent relationship of nascent envelope membranes to the perimeter of viroplasmic domains. In physical models, lipid films may be artificially prepared between interfaces of aqueous compartments (26, 33), and it is tempting to speculate that partitioning of the viroplasmic gel into regions of differing electron densities may reflect the existence of an internal-phase boundary which can orient virus membrane precursors into stable bimolecular layers.

Careful search of vaccinia-infected HeLa cells not treated with rifampin disclosed few and relatively small clusters of envelopes during the period of 4 to 12 hr postinfection; however, large and numerous clusters of envelopes were observed after removal of rifampin from cells infected for 8 hr. Tentatively, we may postulate that the latter experiment has accentuated a stage of vaccinia

TABLE 2. Relative area of membrane-limited domains (MLD) and ratio of grain counts to total area of viroplasm and to area of membrane-limited domains examined in separate sets of autoradiographs^a

Total viroplasm examined (μm^2)	Area of MLD %	Grains/ μm^2	
		Viroplasm	MLD
790	5.8	1.3	0.8
400	4.8	2.5	1.8
520	5.4	2.0	1.5

^a Cells were pulsed with ^3H -thymidine from 2.0 to 2.5 hr after infection in the presence of rifampin and were fixed at 8 hr. Grain densities are comparable only within each experiment, owing to recognized variations in emulsion, exposures, and development (4, 16). Approximately 1,000 grains were counted in each experiment, but the total viroplasm examined differs.

morphogenesis which is normally rapid and transitory. Several investigators have suggested that incomplete envelopes (type I particles) which originate from clusters may enlarge by extension to form complete (type II) immature particles (1, 2, 22). Present observations after rifampin treatment of cells previously infected for 4 to 6 hr suggest that extension of preexisting envelope units can proceed by addition of uncoated membrane "fringes." A similar phenomenon was suggested by the observations of Dales and Mossbach (7) in cells treated with actinomycin D. The evidence is not yet conclusive, since peeling off of the envelope coat might conceivably occur under abnormal conditions.

Formation of a nucleoid in immature vaccinia leads to the appearance of type III particles and appears to be the initial step in core differentiation. Later stages of vaccinia morphogenesis are not observed when nucleoids fail to form (11, 28). Nucleoids rarely develop in the absence of vaccinia DNA replication (15, 28) or when the synthesis of late vaccinia proteins is inhibited (11), but the exact stage in particle morphogenesis at which progeny DNA is incorporated has not yet been determined (14). Under the conditions of rifampin treatment, ^3H -thymidine did not preferentially concentrate in the dense matrix material within membrane-limited domains. The specific autoradiographic labeling of mature particles after removal of rifampin might, therefore, have been due to an influx of DNA after removal of rifampin. The latter possibility could not be verified directly, since the fraction of immature vaccinia particles which were in intermediate stages of maturation was relatively small at any

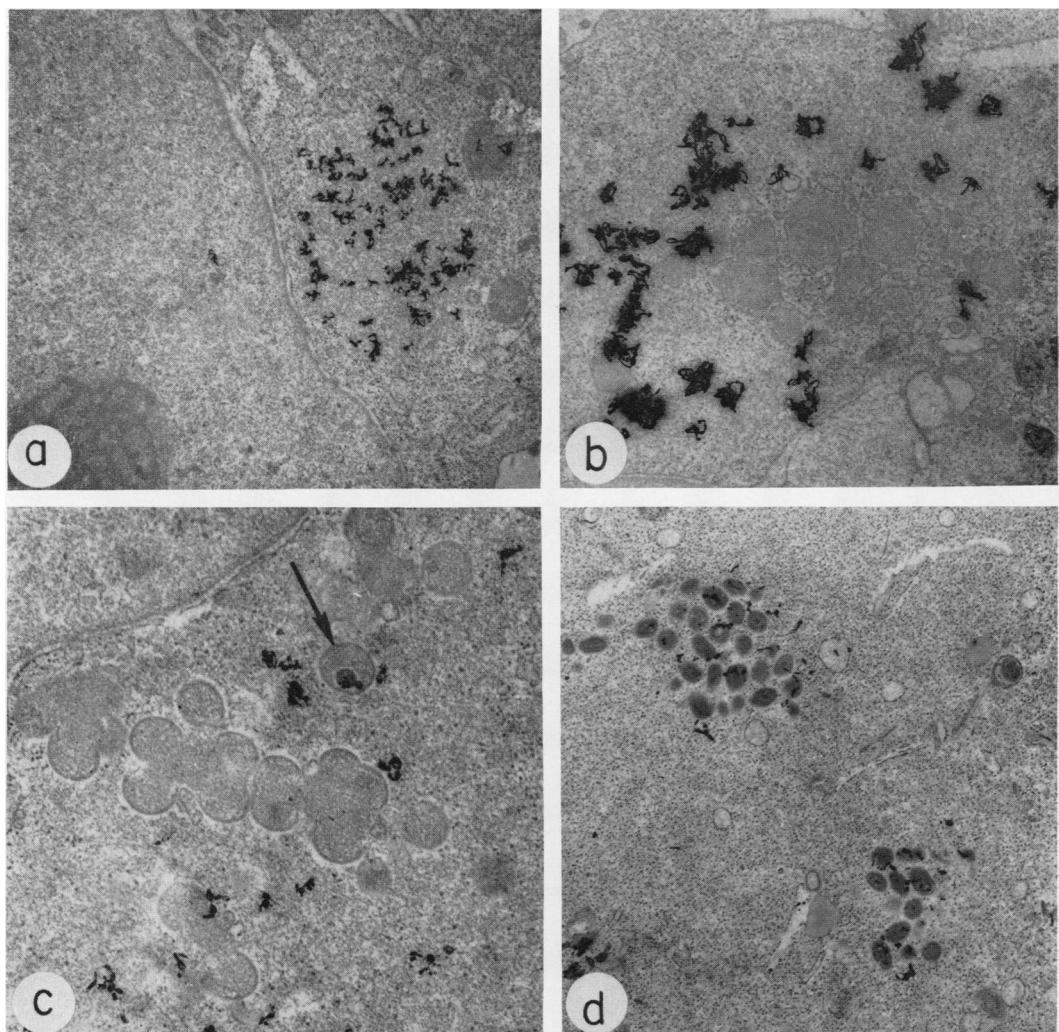


FIG. 11. Autoradiographs of HeLa cells infected with vaccinia virus in the presence of rifampin and pulsed with ^3H -thymidine from 2.0 to 2.5 hr. *a*, Cell fixed at 2.5 hr. Lightly developed silver grains are localized over the viroplasm. $\times 10,000$. *b*, Cell fixed at 8 hr. Fully developed grains do not localize over group of membrane-limited domains. $\times 13,400$. *c*, Cell fixed at 10 min after removal of rifampin (same conditions as Fig. 8). Some grains are associated with a nucleated (type III) particle (arrow). $\times 21,500$. *d*, Cell fixed at 4 hr after removal of rifampin. Lightly developed grains are concentrated above groups of mature vaccinia particles. $\times 13,400$.

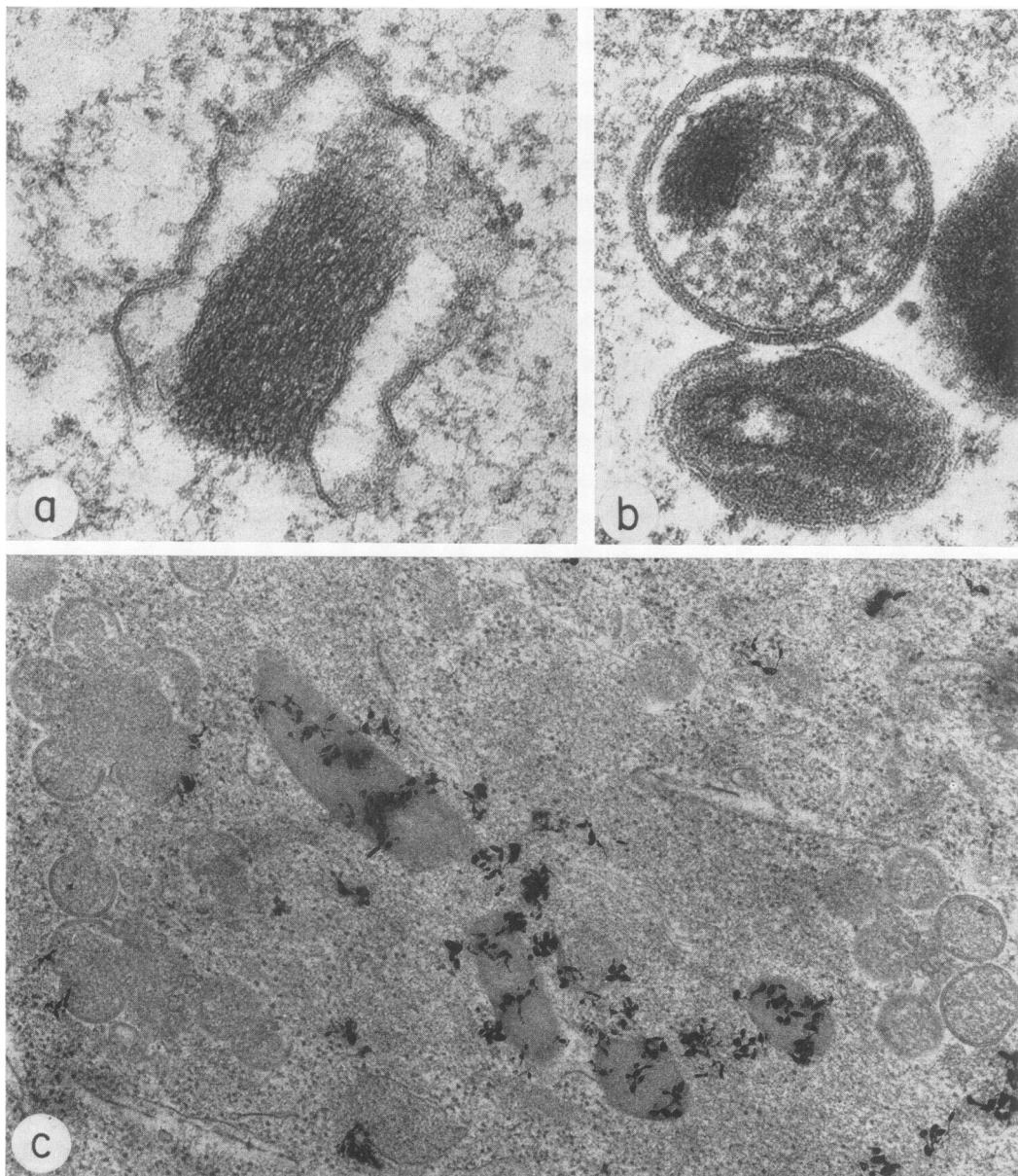


FIG. 12. *a*, High resolution of a dense crystalloid body which formed in a vaccinia-infected HeLa cell during treatment with rifampin (see Fig. 3a). $\times 111,000$. *b*, Similar striate appearance of the nucleoid body in a type III vaccinia particle formed in the absence of rifampin. $\times 121,000$. *c*, Autoradiograph of a crystalloid body which formed during rifampin treatment of a cell pulsed with ^3H -thymidine (same conditions as Fig. 11c). $\times 28,000$.

single time during development, and the size of individual maturing particles is near the practical limits of resolution in electron-microscopic autoradiography (29). The heavy labeling of cytoplasmic crystalloids which are ultrastructurally identical to the nucleoids of immature vaccinia indirectly suggested that vaccinia nucleoids must

also be exceptionally rich in viral DNA and that nucleoid formation may be coincident with DNA concentration in the virus core. It seems logical to speculate that the DNA-rich crystalloids which form during rifampin treatment may represent abnormal precipitates of surplus nucleoid material which was not appropriately integrated due

to the blockage of vaccinia particle assembly. This also appears to occur in L cells (27).

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