

## LOCALIZATION OF NUCLEOTIDE PHOSPHOHYDROLASE ACTIVITY WITHIN VACCINIA\*

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The penetration of infecting vaccinia virus into a host cell occurs in three characteristic stages, culminating in the release of its deoxyribonucleic acid (DNA):<sup>1</sup> (a) the virus is adsorbed to the cell membrane and is then engulfed within a phagocytic vacuole; (b) the envelope of the virus and the membrane of the vacuole are lysed simultaneously, releasing the genome-encapsulating core into the cytoplasm; (c) the coat of the core is then ruptured, allowing the DNA to stream out into the matrix of the cytoplasm. The third stage, termed uncoating, requires the presence of a protein factor which must be formed during the process of penetration.<sup>2</sup> The events of uncoating thus necessitate the existence of a DNA-dependent ribonucleic acid (RNA) polymerase in the core of the vaccinia virus which regulates the transcription of the RNA template for the synthesis of the uncoating protein.<sup>3, 4</sup>

While studying the RNA polymerase activity of vaccinia cores, Kates and McAuslan<sup>4</sup> observed the requirement of an adenosine triphosphate (ATP) regenerating system for optimal RNA synthesis by the vaccinia DNA-RNA polymerase complex. Similarly, Munyon *et al.*<sup>5</sup> noted that 10–100 times more ATP than any of the three other nucleotide triphosphates was necessary for optimal RNA synthesis in this system. These observations suggest, among other possibilities, the presence of an ATP phosphohydrolase associated with the core of the vaccinia virus.

It is of note, however, that vaccinia is a member of the only class of viruses with a lipoprotein envelope, whose biosynthesis is directed by the viral genome<sup>6</sup> rather than being derived from a host cell membrane. Because ATP phosphohydrolases are frequently encountered in biological membranes, a study was undertaken to determine if such an enzyme could be demonstrated in vaccinia and, if so, to determine with which structural component of the virus it is associated.

*Materials and Methods.*—Vaccinia virus was grown in "L" cells and purified.<sup>6</sup> Complete viral purity, as monitored by electron microscopy, was achieved only when the virus pellet obtained by centrifugation through a 50% sucrose cushion was banded on a 20–50% potassium tartrate gradient.

The ATP phosphohydrolase assay was performed with the use of a modification of the procedure described by Pullman<sup>7</sup> that utilizes ATP- $\beta, \gamma$ -<sup>32</sup>P (21–84  $\mu\text{c}/\mu\text{mole}$  in aqueous solution) as substrate. Unless otherwise stated, the assay was carried out for 60 min at 37°C in a reaction mixture prepared to contain 1 mM MgSO<sub>4</sub>, 1 mM KCl, 1 mM NaCl, and 50 mM Tris-SO<sub>4</sub> at pH 7.4. Varying quantities of virus ( $5 \times 10^7$  to  $5 \times 10^8$  PFU) and ATP concentrations (0.06–0.6 mM) were used in different experiments to compensate for isotope decay. Each experiment was compared with a control assay carried out under identical conditions of virus and ATP content. The radioactive inorganic phosphate (<sup>32</sup>P<sub>i</sub>) released by the phosphohydrolase reaction was determined in each sample by mixing 1-ml aliquots of the washed organic phase of the extraction mixture (isobutanol:benzine) with 10 ml of a solution consisting of 5 gm 2,5-diphenyloxazole and 100 gm naph-

thalene dissolved in *p*-dioxane to a volume of 1 liter. The samples were counted in a Beckman LS-200B liquid scintillation system at an efficiency of 95%.

Adenosine diphosphate- $\beta$ - $^{32}\text{P}$  (ADP- $\beta$ - $^{32}\text{P}$ ) and glucose-6-phosphate- $^{32}\text{P}$  (G-6- $^{32}\text{P}$ ) were obtained from ATP- $\beta$ , $\gamma$ - $^{32}\text{P}$  by using the enzyme hexokinase.<sup>8</sup> The effect of the vaccinia phosphohydrolase on the ADP- $\beta$ - $^{32}\text{P}$  and G-6- $^{32}\text{P}$  was tested in the same reaction mixture as that described above.

Rabbit antivaccinia antisera were prepared by immunizing male albino rabbits with vaccinia virus originally grown in embryonic chick fibroblast monolayers. Initial immunization was performed intraperitoneally, followed by periodic intravenous injections of virus over an 8-month period. The antisera employed were found to have high titers of antivaccinia antibodies on both viral neutralization and Ouchterlony testing.

Nonidet P-40 (NP-40) treatment of the virus was carried out for 15 min at 37°C at a final concentration of 0.33% of the nonionic detergent. After treatment, the virus was pelleted by centrifugation at 35,000 *g* for 30 min, and both the pellet and supernatant were studied for phosphohydrolase activity. Where 2-mercaptoethanol (2-ME) was used in conjunction with NP-40,<sup>9</sup> the virus was treated with the detergent as described above and then the 2-ME added to a final concentration of 0.035%. The mixture was then incubated for an additional 10-min period at 37°C. The residual particulate viral structures were pelleted by centrifugation at 35,000 *g* for 30 min, and both the pellet and supernatant were tested for phosphohydrolase activity. In each case the pelleted material was examined as negatively stained whole mounts.<sup>10</sup>

Histochemical studies of the viral phosphohydrolase were carried out by an adaptation of techniques previously described.<sup>11</sup> A purified vaccinia virus preparation and a pellet of vaccinia-infected "L" cells were fixed in 10% calcium-formol for 10 min at 4°C and then exposed to a reaction mixture containing 1 mM ATP, 1 mM  $\text{Pb}(\text{NO}_3)_2$ , 10 mM  $\text{MgSO}_4$ , 8% sucrose, and 0.1 *M* Tris-maleate buffer at pH 7.4. Parallel experiments in which the reaction mixture contained  $10^{-3}$  *M* *p*-hydroxymercuric benzoate (PHMB) were performed. The reacted purified virus was examined in whole mounts. The reacted infected "L" cells were placed in Palade's fixative<sup>12</sup> for 15 min, dehydrated in neutral ethanol series, and processed for thin sectioning.<sup>1</sup>

**Results.**—Figure 1 shows the ATP phosphohydrolase activity of the purified vaccinia virus preparation under the experimental conditions employed. The

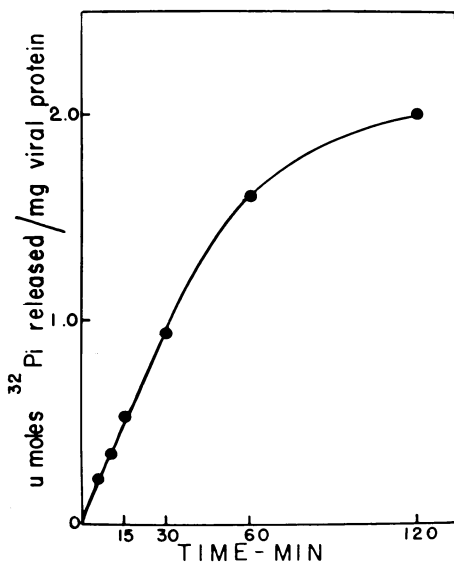


FIG. 1.—Release of  $^{32}\text{P}_i$  from ATP- $\beta$ ,  $\gamma$ - $\text{P}^{32}$  by purified vaccinia. Reaction mixture (200  $\lambda$ ) contained  $5 \times 10^8$  PFU of vaccinia and 0.6 mM ATP. Amount of viral protein was estimated from the number of PFU (see text.)

rate of  $^{32}\text{P}_i$  release was linear for the first 60 minutes but decreased thereafter. Based on average values of 100 elementary vaccinia virus particles per PFU in our clumped material, and an estimated value of  $5.35 \times 10^{-9}$   $\mu\text{g}$  of protein per virus particle,<sup>13</sup> it was calculated that the amount of phosphohydrolase enzyme in 1 mg of vaccinia virus protein would hydrolyze 1.6  $\mu\text{moles}$  of ATP in 60 minutes under the experimental conditions employed.

The purified vaccinia preparation is shown in Figure 2, and the effect of NP-40 alone on the viral structure is shown in Figure 3. Treatment with the detergent caused the membrane to unfold, allowing penetration by stain, but the particle remained otherwise intact. However, after the addition of 2-ME, complete removal of the viral membrane occurred, leaving the core and lateral bodies behind (Fig. 4).

The effects of various forms of virus treatment upon the phosphohydrolase activity is shown in Table 1. Heating at 80°C for ten minutes decreased the

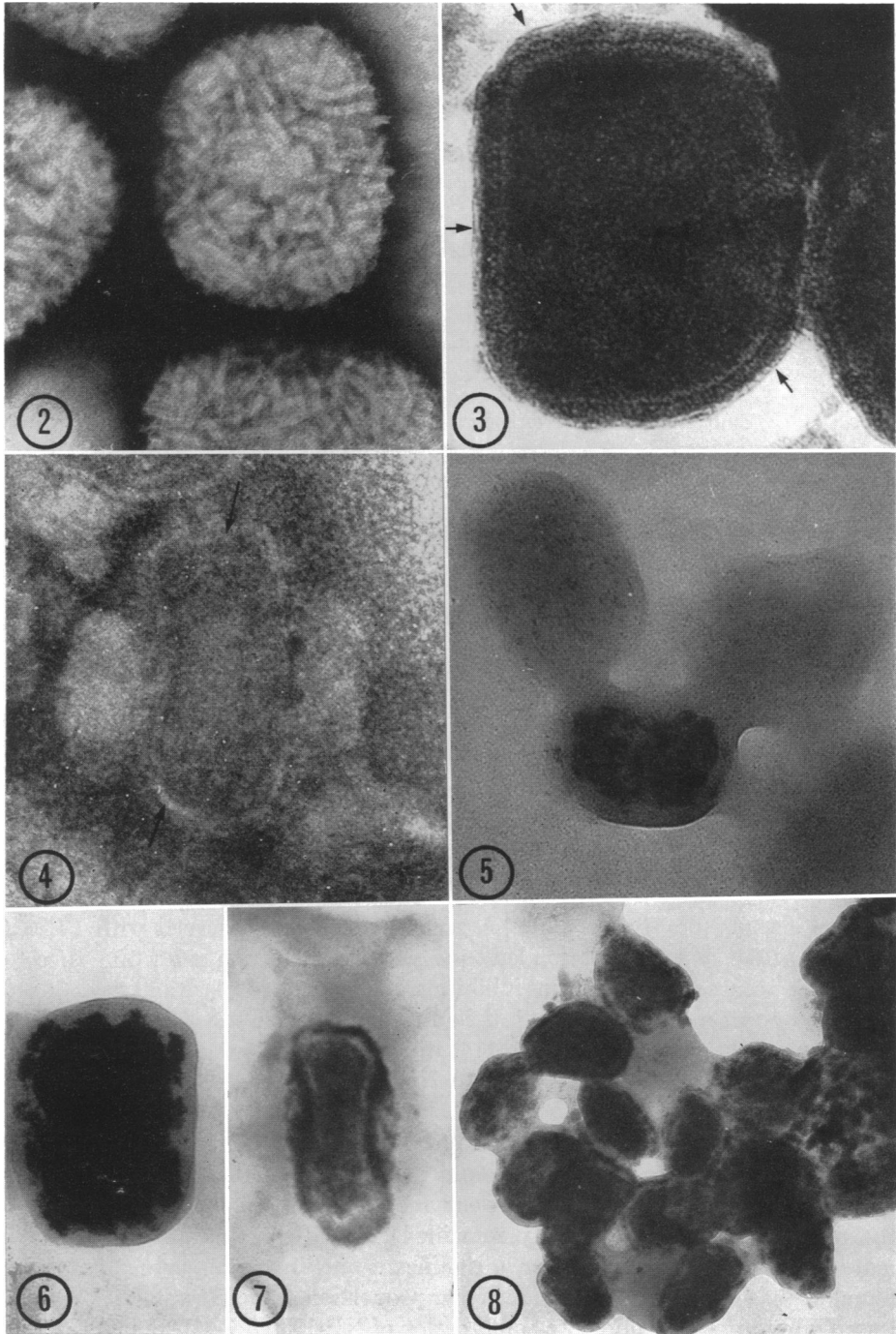
TABLE 1. *Effect of virus treatment on the nucleotide phosphohydrolase activity.*

Viral treatment	$^{32}\text{P}_i$ released (%) compared with control*
None—control	100
Heated 80°C, 10 min	4
Ouabain, $10^{-5}$ M	103
Ouabain, $10^{-3}$ M	100
Preimmune rabbit serum (diluted 1:4)	102
Rabbit antivaccinia antiserum (diluted 1:4)	104
PHMB, $10^{-5}$ M	13
PHMB, $10^{-2}$ M	4
Nonidet P-40	
Pellet	208
Supernatant	53
Nonidet P-40 and 2-mercaptoethanol	
Pellet	201
Supernatant	107
Calcium formol (10%)	102

\* In every case the experimental assay was compared with a control containing identical quantities of untreated virus and ATP- $\beta$ ,  $\gamma$ - $^{32}\text{P}$ .

enzyme activity by 96 per cent. A graded effect was observed with PHMB whereby a  $10^{-5}$  M concentration led to 87 per cent inhibition and a  $10^{-2}$  M concentration resulted in 96 per cent inhibition of phosphohydrolase activity. Ouabain at concentrations up to  $10^{-3}$  M had no effect upon the enzyme function. Similarly, rabbit antiserum (diluted 1:4) which demonstrated a 99.9 per cent viral neutralizing ability at a dilution of  $10^{-2}$  showed no inhibition of phosphohydrolase activity.

The effects of NP-40 alone and that of NP-40 in conjunction with 2-ME were similar with regard to the function and distribution of the vaccinia phosphohydrolase enzyme. In both cases the enzyme activity was notably enhanced, and the major proportion of activity remained associated with the pelleted viral material. In each case, however, a significant amount of enzyme activity was found in the supernatant fluid. A higher proportion of enzyme activity was released into the supernatant by a combination of NP-40 and 2-ME than by NP-40 alone.



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Modification of the reaction mixture, as outlined in Table 2, revealed a number of properties of the viral phosphohydrolase. Omission of  $\text{Na}^+$  and  $\text{K}^+$  ions from the reaction mixture failed to influence enzyme function. The presence of unlabeled nucleotides in the assay medium served to inhibit the release of  $^{32}\text{P}_i$  from the  $\text{ATP-}\beta,\gamma\text{-}^{32}\text{P}$ . The nucleotide triphosphates of adenosine (ATP), guanine (GTP), inosine (ITP), and uridine (UTP) performed this function in decreasing order of efficiency. The presence of unlabeled inorganic phosphate in the reaction mixture was also inhibitory to the phosphohydrolase activity.

TABLE 2. *Effect of modifications of the reaction mixture on the viral nucleotide phosphohydrolase activity.\**

Modification of reaction mixture	$^{32}\text{P}_i$ released (%) compared with control
None—control	100
$\text{Na}^+$ and $\text{K}^+$ ions omitted	102
Unlabeled nucleotides added	
ATP, 0.2 mM	36
GTP, 0.2 mM	40
ITP, 0.2 mM	67
UTP, 0.2 mM	88
Inorganic phosphate (1 mM) added	48
Unlabeled ADP added	
0.06 mM	97
0.6 mM	95
6.0 mM	95

\* In this series of experiments, each reaction mixture contained  $10^8$  PFU of vaccinia and an  $\text{ATP-}\beta,\gamma\text{-}^{32}\text{P}$  concentration of 0.1 mM.

Studies of the action of the virus on ADP revealed that relatively massive quantities of unlabeled ADP did not compete with  $\text{ATP-}\beta,\gamma\text{-}^{32}\text{P}$  for enzyme action. This result was confirmed by the finding that the viral enzyme failed to release  $^{32}\text{P}_i$  from  $\text{ADP-}\beta\text{-}^{32}\text{P}$  or  $\text{G-6-}^{32}\text{P}$ .

Calcium-formol failed to affect enzyme function (Table 1), thereby allowing this reagent to be used as a fixative for the histochemical studies.

The results of the histochemical studies are shown in Figures 5–10. Examination of vaccinia whole mounts revealed histochemical staining localized completely to the virus core. In thin sections of infected “L” cells, the reaction product for the phosphohydrolase enzyme was present inside immature particles, but was absent from mature progeny. The mitochondria also showed heavy

FIG. 2.—Purified virus from tartrate gradient: whole mount negatively contrasted with phosphotungstic acid.  $\times 144,000$ .

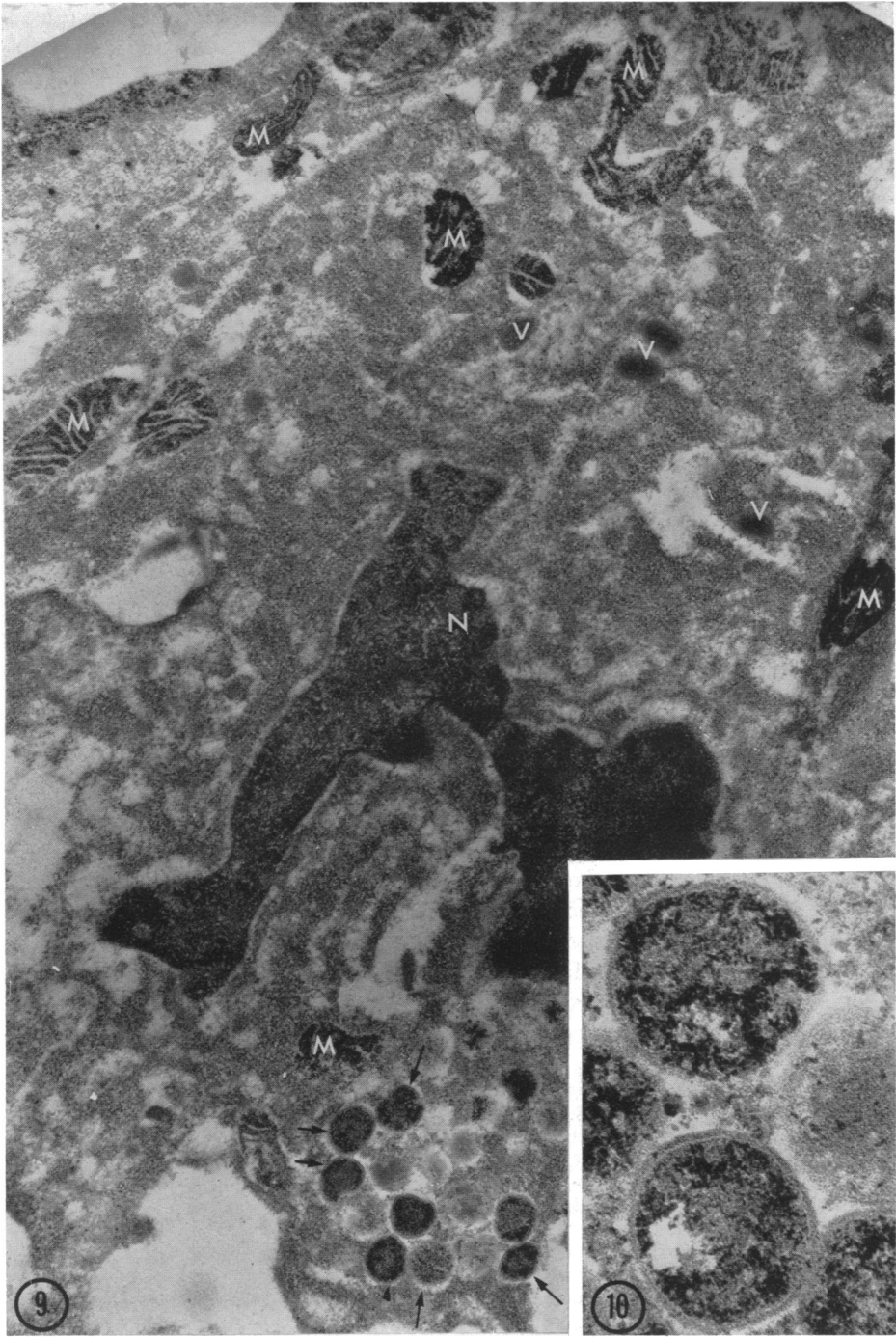
FIG. 3.—As above, after treatment with Nonidet P-40. Arrows indicate the remaining membrane.  $\times 180,000$ .

FIG. 4.—As above, after treatment with detergent and 2-mercaptoethanol. Note two lateral bodies in contact with a residual core (arrows).  $\times 153,000$ .

FIG. 5.—Whole mount of four particles after incubation in reaction mixture with PHMB. Note the general paucity of product. The group selected represents the most intense reaction observed in three separate experiments. Usually, product was completely absent.  $\times 90,000$ .

FIGS. 6 and 7.—As above, without PHMB. Intense staining of the core is evident in particles displayed in their broad and narrow aspect. Fig. 6,  $\times 108,000$ ; Fig. 7,  $\times 91,800$ .

FIG. 8.—An aggregate of particles as in Figs. 6 and 7, illustrating the high frequency of staining in the population of particles.  $\times 62,100$ .



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deposition of reaction product (Fig. 9). Treatment of purified virus suspensions or vaccinia-"L" cell complexes with PHMB served to inhibit the deposition of the reaction product in both the virus and mitochondria.

*Discussion.*—The results show that vaccinia contains a nucleotide phosphohydrolase associated exclusively with the viral core. This enzyme is apparently not specific since the presence of other nucleotides interferes with the phosphohydrolysis of ATP. Unlike the cellular membrane<sup>14</sup> and microsomal<sup>15</sup> ATP phosphohydrolases, the viral enzyme neither requires Na<sup>+</sup> and K<sup>+</sup> ions for its activity, nor is it sensitive to ouabain. It is presumably not a phosphatase since it fails to interact demonstrably with ADP or G-6-<sup>32</sup>P.

The foregoing results are in agreement with those recently reported by Munyon *et al.*<sup>16</sup> who found similar properties for the vaccinia phosphohydrolase and also demonstrated the lack of stimulation of the viral enzyme by Ca<sup>++</sup>. This result, they concluded, distinguishes the viral phosphohydrolase from mitochondrial ATPase.<sup>17</sup> They did report, however, that ouabain led to a 27 per cent inhibition of nucleotide phosphohydrolase activity in their vaccinia preparation. Since the virus suspension which they employed was obtained after banding on sucrose gradients only, it is possible that their preparation was contaminated by minute quantities of cellular debris containing a ouabain-sensitive phosphohydrolase. This would account for the discrepancy between their results and ours.

The first indication that this enzyme might not be a membrane constituent was the finding that antivaccinia antiserum of high neutralizing titer had no effect on the enzyme activity. Then treatment of the virus with NP-40 and 2-ME revealed that most, but not all, of the markedly enhanced phosphohydrolase activity remained associated with the pelleted viral cores. Whether the solubilized membrane carried some phosphohydrolase activity into the supernatant or whether some of the core enzyme had been released into solution by treatment of the virus could not, of course, be determined from this result.

The histochemical studies clarified this situation. Whole mounts of vaccinia showed that the product of phosphohydrolase activity had formed specifically in the core. By means of thin sections it was ascertained that immature progeny contained reaction product scattered throughout the interior of the particles, but not on or near the membranes. In fact, the histochemical reaction was found to have occurred in some immature particles before a complete membrane had been formed. In particles which had differentiated further and contained the DNA nucleoid, the product formed at the periphery of this structure.

The failure to demonstrate reactivity of mature particles located within the host cell suggests that the mature virus, while still in association with intracellular membranes, is impervious to the substrate or other components of the reaction mixture. This implies that the envelopes of vaccinia preparations that

FIG. 9.—Thin section of an infected cell exposed to histochemical reaction mixture. Irregularly shaped nucleus (*N*) occupies the center. Mitochondria (*M*) and immature virus (*arrows*) show intense activity, whereas mature progeny (*V*) are devoid of stain.  $\times 27,840$ .

FIG. 10.—Immature progeny from a specimen as in Fig. 9 illustrate the occurrence of product inside and its absence from the viral envelope.  $\times 95,700$ .

have undergone purification are altered and become permeable, thus allowing reaction product to accumulate in the core. If this assumption is correct, it raises the possibility that certain of the chemically measured enzyme functions, such as the rate of enzyme reaction, may well reflect the degree of membrane damage incurred during viral purification.

We now consider briefly the possible function of a nucleotide phosphohydrolase associated with the vaccinia core. Because of its location within the virus, it is not likely to act during the early stages of penetration and membrane lysis. Since optimal activity of the viral DNA-RNA polymerase system, apparently active in the uncoating process, requires high concentrations of ATP or an ATP-regenerating system, the availability of an ATP phosphohydrolase for this function appears antithetic.

It may be that the viral phosphohydrolase enzyme is present in order to make phosphate groups available for other, yet unknown viral functions. Finally, this enzyme may act by forming an intimate association with certain strands of progeny DNA when they are about to become packaged within envelopes. The presence of the nucleotide phosphohydrolase in this position would lead to hydrolysis of the terminal phosphate groups of incoming nucleotides which are susceptible to the action of the enzyme (see *Note added in proof*). In this way the enzyme could regulate the cessation of DNA replication and transcription.

*Summary.*—It was demonstrated that vaccinia contains a nucleotide phosphohydrolase enzyme with affinities for a number of nucleotide triphosphates. By the use of histochemical procedures the enzyme function was localized to the core of the virus.

*Note added in proof:* Preliminary studies indicate that deoxyribonucleotide triphosphates compete with ATP in the viral phosphohydrolase reaction with greater efficiency than the corresponding ribonucleotide triphosphates.

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