*TWO DEOXYRIBONUCLEASE ACTIVITIES WITHIN PURIFIED VACCINIA VIRUS**

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Abstract.—Two DNases, both hydrolizing single-stranded DNA, were identified within highly purified particles of vaccinia virus. One of these, active optimally at pH 5.0, is an exonuclease and the other, most active at pH 7.8, is an endonuclease. These two activities were localized more specifically within the virus cores. Upon elimination of the envelopes, followed by removal of the lateral bodies of vaccinia, both DNases were activated, suggesting that a specific inhibitor of these enzymes may be present in the lateral bodies.

Introduction.—Although discovery of the first virus-associated enzyme, the neuraminidase of myxoviruses, was reported over 25 years ago,[1] existence of other enzymes within virus particles was established only recently. One enzyme detected in reoviruses is an RNA transcriptase that is intimately associated with the double-stranded RNA genome.[2] During penetration and uncoating, this enzyme presumably passes into the cytoplasmic matrix and starts to function while still attached to the parental genomes. In another group of agents, the poxviruses, two enzymes have been detected thus far, a DNA-dependent RNA polymerase[3] and a nucleotide phosphohydrolase.[4, 5] Both activities are present as an integral part of the viral cores. During penetration into host cells, the cores and associated enzymes become released into the cytoplasmic matrix where they can function while the parental DNA genome is still enclosed within the core.[3, 4] It now appears certain that inoculum particles of reo- and poxvirus carry into the host enzymes required for the early steps in replication. As a result of continued search for other activities integrated within vaccinia that could participate in the early phases of infection, two deoxyribonucleases were detected. This study deals with the localization and properties of these two factors.

Materials and Methods.—Vaccinia virus was grown in L cells, purified, and examined by electron microscopy as previously described.[6] Methods for obtaining antisera have also been described.[4] Controlled stripping of the envelopes and removal of lateral bodies was performed by modified procedures of Easterbrook.[7] Clumped purified vaccinia were resuspended in 0.01 *M* Tris buffer, pH 7.3, and dispersed by a 10-sec sonication with a Branson sonifier. Envelopes were stripped by incubating for 15 min at 37° with 0.5% Nonidet-P_{40} detergent (NP_{40}) in Tris buffer, then for 10 min with 0.25% of 2-mercaptoethanol in Tris buffer. Stripped virus was concentrated through layers of 20% and 50% sucrose in a Spinco SW39 rotor and spun for 20 min at 25,000 rpm. Lateral bodies were digested by exposing 100-μg aliquots of stripped virus to 20 μg of trypsin (final vol 0.5 ml) and resulting cores concentrated in the same manner as stripped virus.

The DNA substrate was obtained from L cells grown for 24 hr in nutrient medium containing 0.5 μCi/ml of thymidine methyl-^{3}H (spec. act. 11 Ci/mM). Cell pellets were suspended in 0.25 *M* sucrose and 10^{-2} *M* KCl, then disrupted by a Dounce homogenizer. To extract the DNA, freed nuclei were spun into pellets at 1000 rpm for 6 min, re-

suspended, and treated in sequence with sodium dodecyl sulfate, RNase, and pronase.[8] DNA fibers obtained by this procedure were dissolved in 0.15 *M* NaCl and separated into single strands by heating for 5 min at 100° and cooled rapidly. Purity of the DNA was checked by (1) UV spectrophotometry using absorption ratios at 260 vs. 280 and 260 vs. 235 mμ, and (2) Burton's[9] deoxyribose determination. Ratio of values for deoxyribose to UV absorption was 1.2. Hydrolysis of DNA by the purified virus was determined with the procedures employed with infected cells.[10] Four buffers, each at a final concentration of 0.5 *M*, provided the desired range of pHs: acetate–acetic acid for 3.6–5.6; phosphate for 6 to 7; Tris–HCl for 7–9; glycine–NaOH for 9–10. 0.25% NP_{40} and 20 mM 2-ME† were included in the incubation mixture. In each case, controls with viral material omitted were run concurrently. Hydrolysis was stopped after adding carrier protein (albumin) and cold perchloric acid to a final concentration of 0.5 *N*. The resulting precipitate was removed by centrifuging for 30 min at 3000 rpm and the amount of PCA-soluble tracer measured with a 25% efficiency in a Beckman scintillation counter, with scintillation fluid mixtures of naphthalene, 2,5-diphenyloxazole, and *p*-dioxane.[4] The respective DNase activities were characterized as *exo-* or *endonuclease* by means of a filter assay.[11]

Result.—(1) Characterization of DNase activity: After detection of DNase activity in highly purified vaccinia particles, it was necessary to ascertain which among the three activities that appear in host cells after infection[10] was contained within the virus. As with the induced cell-associated DNases, the activity contained in virus particles hydrolized exclusively denatured DNA, while native DNA was entirely resistant (Fig. 1). Heating the virus for five minutes at 80° completely abolished hydrolysis. Incubation of virus with melted DNA in a pH range of 3.6 to 10 revealed activity at two distinct pH optima, 5.0 and 7.8 (Fig. 1): suggestive evidence for the presence of two enzymes. Additional supportive data were obtained by the following experiments.

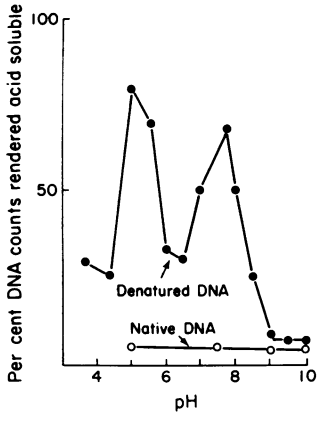

FIG. 1.—Relationship between DNA hydrolysis and pH. Each 0.1–0.2 ml sample contained NP_{40}, 2-mercaptoethanol, 10–20 μg of vaccinia protein, 6 μg of DNA (8000 cpm), and the appropriate buffer. The mixture was incubated at 37° for 1 hr. Values are averages of 3 independent experiments.

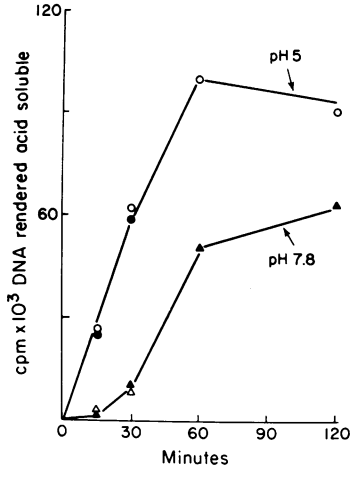

FIG. 2.—Time-course of DNA hydrolysis at two pH's. Incubation mixture, as in Fig. 1, contained 5 μg DNA (12,000 cpm). Duplicate samples preincubated without substrate in NP_{40} and 2-mercaptoethanol for 15 or 30 min were also tested. Average values from two experiments.

The rate of DNA hydrolysis as a function of time was different at pH 5.0 and 7.8. In each case, the reaction was run at saturating substrate concentrations. Although the concentration of the virus-associated enzymes was unknown, the differences observed in the rate of hydrolysis suggest two types of activities. From other data based on the relationship between velocity and substrate concentration, the substrate affinity (K_m) of the two activities was calculated by means of the Lineweaver-Burk plot.[12] The reaction run at pH 5.0 had a K_m of 9.1×10^{-6} gm of DNA, and the reaction carried out at pH 7.8 a K_m of 3.2×10^{-5} gm of DNA, indicating higher substrate affinity of the acid DNase. Preincubation of virus in NP_{40} and 2-ME failed to abolish the lag period observed consistently at pH 7.8 (Fig. 2). Another means of distinguishing the two DNases was based on the position of the internucleotide linkage susceptible to hydrolysis. Determination of the rate of DNA breakdown into fragments soluble in PCA revealed that the pH 5.0 activity is an exonuclease (Fig. 3) and pH 7.8 activity an endonuclease (Fig. 4). A third approach for differentiating these two activities involved the addition of various cations, anions, and transfer RNA (tRNA). The results, summarized in Table 1, showed that $PO_4^{=}$ inhibited DNase activity at pH 5.0 more than at 7.8 while Mg^{++}, Ca^{++}, Na^{+}, K^{+}, and NH_4^{+} were relatively more inhibitory at pH 7.8. At pH 5.0 tRNA proved to be a more effective, competitive inhibitor of the reaction than at pH 7.8 (Table 1). Further evidence for a viral specificity of these nucleases was provided by experiments in which addition of antivaccinia serum to the incubation mixture inhibited DNA hydrolysis (Table 1). However, since fetal bovine serum and preimmune rabbit serum also reduced partially both the DNAse activities, the true measure of the specific inhibition by the antibody itself was not determined. Data from several independent experiments also showed that antiserum was less effective against the activity at pH 7.8.

(2) *Localization of DNase activity within the virus:* Mature vaccinia virus

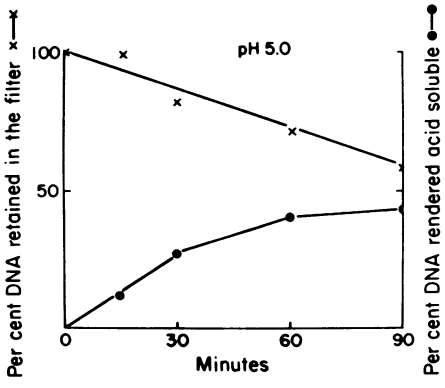

FIG. 3.—Rates of DNA either rendered acid-soluble or retained on the filter. Incubation mixture described in Fig. 1, contained 10 μg of vaccinia protein and 2 μg of DNA (18,000 cpm). Average of 2 experiments.

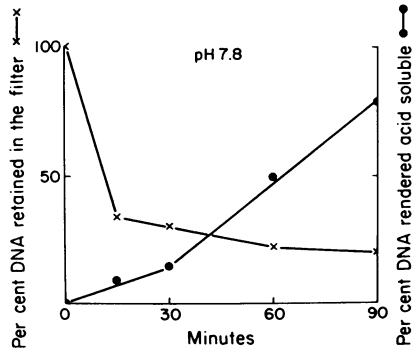

FIG. 4.—Rates of DNA either rendered acid-soluble or retained on the filter. Incubation mixture described in Fig. 1, contained 20 μg of vaccinia protein and 4 μg DNA (36,000 cpm). Average of 2 experiments.

TABLE 1. *Relative activities of two DNases in the presence of various salts, antiserum, and transfer RNA.*

Modification of reaction mixture	pH 5.0 DNase (% of control)	pH 7.8 DNase (% of control)
$MgCl_2$	100	20
$CaCl_2$	35	15
$MnCl_2$	100	100
NaCl	100	50
KCl	100	55
Na_2PO_4	46	67
$KHPO_4$	52	100
$(NH_4)_2SO_4$	100	60
Na_2SO_4	45	50
NH_4Cl	100	57
Fetal bovine serum 1:5	30	100
Preimmune rabbit serum 1:5	85	60
Rabbit antiserum 1:5	18	40
Transfer RNA 10 μg	50	100
Transfer RNA 20 μg	30	50
Transfer RNA 50 μg	30	50

The basic incubation mixture described in Fig. 1 contained also, where appropriate, 1 mM of each salt. The tRNA from *E. coli* was kindly supplied by Dr. I. Smith. The results, expressed as percentage of activity in the absence of additive, are based on the micrograms of denatured DNA hydrolyzed per milligram viral protein per 30 min at pH 5.0 and per 60 min at pH 7.8.

consists of three major components: (*a*) an outer and lipoprotein envelope, (*b*) a flat and biconcave inner core, and (*c*) two lateral dense masses, the so-called lateral bodies (Fig. 5*A* and *B*). When the envelope is stripped under controlled conditions, the two lateral bodies remain, apparently intact (Fig. 5*C*). The lateral bodies, in their turn, can be digested specifically with trypsin, leaving behind the core structure (Fig. 5*D*). Localization of the DNases was accomplished by assaying whole virus, particles stripped of envelopes, and purified isolated cores for activity at pH 5.0 and 7.8. The results summarized in Table 2 showed that untreated, whole virus possessed minimal DNase activity. After adding NP_{40} and 2-mercaptoethanol, DNA hydrolysis was consistently enhanced while 2-mercaptoethanol alone was less effective in this respect (Table 2). Particles lacking envelopes, when incubated either with or without NP_{40} and 2-mercaptoethanol, were invariably more active than whole virus. In each case, maximal enhancement was recorded with cores obtained after the digestion of the lateral bodies (Table 2). When cores were used, potentiation of catalytic activity to maximum values did not require the presence of either NP_{40} or 2-mercaptoethanol in the incubation mixture. From the calculated ratios of DNase activity in cores and stripped particles, it became clearly evident that removal of the lateral bodies enhanced DNase activity. The average increase at pH 5.0 was ×5.0, and at pH 7.8 ×11.4 (Table 2). In one experiment at pH 7.8, ×17 stimulation was obtained. A correlation appeared to exist between the digestion of the lateral bodies and the increase of DNase activity.

The time course of the reactions was also tested with isolated cores to determine whether it differed from that found with whole disrupted particles. The results showed that the rate of the reaction at pH 5.0 was unchanged, while at pH 7.8 the characteristic lag period (see Fig. 2) disappeared.

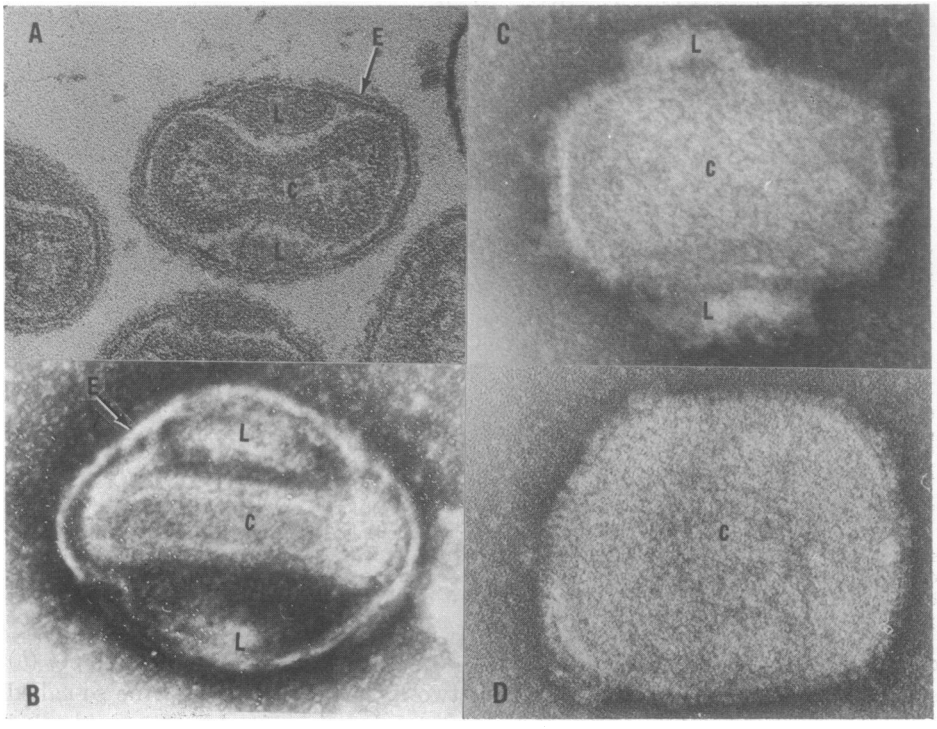

FIG. 5.—Morphology of vaccinia virus as observed in (*A*) thin section or negatively stained preparation of (*B*) whole particle, (*C*) particle stripped of its envelope, (*D*) isolated core. *E*, envelope; *L*, lateral body; *C*, core. ×165,600.

The affinity of the viral DNA hydrolases for their substrate in whole particles or isolated cores was also tested. Reactions were run at pH 5.0 and 7.8 with whole virus and cores, using concentrations of DNA ranging from 2 to 32 μg. Although, as anticipated, the DNA hydrolysis with cores was higher at all substrate concentrations, K_m values calculated according to the Lineweaver-Burk plot were identical with those cited above. These results are interpreted to show that the inhibitor of the DNases acted noncompetitively in the reaction.

Discussion.—All our current observations support the view that vaccinia contains two enzymes able to hydrolyze single-stranded DNA. The two factors are distinguishable one from the other in respect to their pH optima, K_m values, site of nucleolytic attack of the substrate, and activity in the presence of various anions, cations, and transfer RNA. The DNase that acts optimally at pH 5.0 is an exonuclease having properties virtually identical with those reported by McAuslan and Kates for an enzyme that appears in HeLa cells infected with rabbit pox.[10] We are of the opinion that this virus-induced DNase becomes integrated in progeny particles. The second DNase acting optimally at pH 7.8 is an endonuclease, presumably related to the factor appearing after infection of HeLa cells by rabbit pox[13] that also must become integrated within progeny particles. A third alkaline DNase induced after rabbit pox infection[10]

TABLE 2. *Hydrolysis of DNA by whole virus and subviral components.*

Additions	Expt. no.	Whole virus A	Stripped virus B	Cores only C	C:B
		pH 5.0 Activity*			
None	1	760	2,040	5,440	5.0
	2	304	1,240	9,600	
	3	96	188	3,200	
	4	8	320	480	
NP_{40} + 2-mercaptoethanol	1	1,460	6,000	5,640	1.8
	2	800	2,000	12,000	
	3	2,880	3,800	3,800	
	4	200	760	1,480	
	5	2,640	...	8,000	
2-Mercaptoethanol	1	960	3,720	6,440	1.8
	2	360	1,080	...	
	3	...	...	...	
	4	...	760	1,560	
		pH 7.8 Activity*			
None	1	520	520	8,400	11.4
	2	240	400	4,400	
	3	48	80	140†	
	4	8	240	1,240	
NP_{40} + 2-mercaptoethanol	1	1,420	3,720	9,460	2.7
	2	660	1,480	4,960	
	3	440	400	2,440	
	4	280	1,140	1,740	
	5	1,720	...	7,920	
2-mercaptoethanol	1	...	640	8,400	5.6
	2	520	480	...	
	3	...	...	...	
	4	...	1,200	1,920	

Incubation mixture as in Fig. 1.

* Results from four or five independent experiments. The data are expressed as micrograms of denatured L cell DNA hydrolyzed per mg viral protein per hour.

† In expt. 3, removal of the lateral bodies was incomplete and may account for this unusually low value.

was not found by us in particles of vaccinia. The observed reproducible inactivation of the vaccinia DNases by antisera was not unexpected since whole purified virus was used for immunization. The extent of the specific inhibition was, however, not established because spurious inactivation data with control sera were also obtained.

A stepwise dissection of whole, purified virus enabled us to localize both DNases with the viral core. All four enzymes known to exist in poxvirus particles, including the RNA polymerase[3] and nucleotide phosphohydrolase,[4, 5] identified previously, are associated with the core and, furthermore, are all late viral functions (in preparation). Therefore, vaccinia proteins synthesized from late template RNA that is transcribed from the progeny DNA, including enzymes in the core, can be packaged inside viral lipoprotein envelopes whose morphogenesis is regulated by early functions.[6]

Following treatment of purified whole or stripped virus with detergent and mercaptoethanol, the rate of DNase activity increased, indicating that the envelope acts as a barrier preventing interaction of the substrate with enzymes

located deeper within the particle. Additional enhancement of activity by NP_{40} and 2-ME, when added to stripped virus, could mean that after stripping not all the active sites are fully exposed (Table 2). The dramatic increase in the activity of both DNases, observed following digestion of the two lateral bodies (Table 2) could result from increase in substrate accessibility or, as seems most likely, from the removal of a specific inhibitor. In this connection it should be mentioned that an inhibitor of pancreatic DNase has been identified.[14] By contrast, nucleotide phosphohydrolase activity, also present in the core, is unaffected by the presence or absence of the lateral bodies (unpublished observations). It is significant that during virus penetration, the two lateral bodies become separated from the core at the time vaccinia escapes out of the phagocytic vacuole into the cytoplasmic matrix.[15] One can speculate that this event represents the natural process for activating the DNases of the invading vaccinia. Although the role of the RNA polymerase activity in the core has been elucidated,[3] to date we lack definitive information about the other three enzymes. By analogy with other biological systems, enzymes possessing the properties of vaccinia DNases might participate in DNA replication[16] and, like the RNA polymerase, could act during the initial phases of viral reproduction.

In addition to any function that the two DNases may provide for vaccinia, their possible role in turning off cellular macromolecular synthesis deserves further attention. It is well known that poxviruses depress DNA and protein synthesis of the host very promptly (refs. 17–19 and own unpublished observations) and RNA synthesis more gradually.[20] Inoculum particles rendered noninfectious by extensive UV irradiation and those that are prevented from uncoating are as efficient in this respect as viable particles. However, contrary to the situation encountered with some bacteriophages, vaccinia infection does not bring about breakdown of preformed host DNA. It remains to be tested whether DNases released from inoculum particles enter the nucleus and hydrolyze the nascent single-stranded DNA molecules that are formed at the replicative point.[21, 22]

Summary.—An exonuclease active optimally at pH 5.0 and an endonuclease active at pH 7.8, both hydrolyzing single-stranded DNA, were identified in cores of vaccinia virus. Upon elimination of the lateral bodies, both DNases were activated, suggesting that a specific inhibitor of these enzymes may be present in the lateral bodies.

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† 2-Me, 2-mercaptoethanol.

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