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Physicochemical Properties of Fowlpox Virus Deoxyribonucleic Acid and Its Anomalous Infectious Behavior

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ABSTRACT

RANDALL, CHARLES C. (University of Mississippi, Jackson), LANELLE G. GAFFORD, RICHARD L. SOEHNER, AND JAMES M. HYDE. Physicochemical properties of fowlpox virus deoxyribonucleic acid and its anomalous infectious behavior. J. Bacteriol. **91**:95–100. 1966.—Deoxyribonucleic acid (DNA) was extracted from fowlpox virus-infected tissue, purified inclusions, and purified virus by five variations of detergent and phenol methods. Phenol methods gave a poor yield, whereas detergent techniques extracted up to 78% of the DNA. The buoyant density was 1.695 g/ml, and the melting temperature in 7.2 M $NaClO_4$ was 39 C, both approximately equivalent to a guanine plus cytosine content of 35 moles per cent. Further proof of the double-stranded nature of the DNA was shown by the characteristic behavior toward deoxyribonuclease, formaldehyde, and heat. Infectious DNA was obtained by the various methods described, but this manifestation of biological activity was capricious and for unknown reasons was often not evident. The infectivity could not be related quantitatively to the amount of DNA employed. Furthermore, the infectious nature of fowlpox virus DNA was demonstrable only when the route of infection was the chorioallantoic membrane. In contrast, whole virus infected both membrane and chick skin with equal efficiency.

Studies in this laboratory (11) have demonstrated that the nucleic acid of fowlpox virus is deoxyribonucleic acid (DNA) with a guanine plus cytosine (GC) content of approximately 35%. Collaborative studies with Szybalski et al. (16) have shown that native double-stranded DNA could be extracted from purified inclusions and virus. The GC content calculated from the melting temperature (T_m) and from buoyant-density determinations was in agreement with the GC values reported by this laboratory.

In a preliminary note (Randall, Gafford, and Soehner, Federation Proc. **23**:295, 1964), the extraction of subviral infectious material by detergent and phenol methods was described. The infectious nature of the DNA extracts was demonstrated to be specific for the chorioallantoic membrane (CAM).

The present study reports several methods of extraction of fowlpox virus DNA, which has been further characterized by some physicochemical methods. Some of the purified DNA extracts are infectious, but others, similarly prepared, do not exhibit this capacity.

The only other paper dealing with the infectious nature of poxvirus DNA is that of Abel and Trautner (1), in which a subviral entity, essentially vaccinia DNA, was shown to be replicated in competent *Bacillus subtilis*.

MATERIALS AND METHODS

Infected tissue and source of virus. The strain of fowlpox virus, method of inoculation of chick scalp, preparation of infected tissue, isolation of follicles and inclusions, and purification of virus from sonically treated inclusions have been described (11). The isolation of inclusions, matrix, and fowlpox virus followed procedures outlined by Randall et al. (12).

Chemical procedures. Protein and DNA were determined by methods described previously (11).

Enzymes. Deoxyribonuclease, 1× crystallized, and salt-free trypsin, 2× crystallized, were obtained from Worthington Biochemical Corp., Freehold, N.J. Salt-free ribonuclease, 5× crystallized, was the product of Mann Research Laboratories, New York, N.Y.

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Sodium lauryl sulfate (SLS) extraction. Viral particles, inclusions, and matrix or ground follicles were suspended in 10 volumes of saline-citrate (SSC; 0.15 M NaCl plus 0.02 M trisodium citrate), and 1 volume 20% SLS (USP; Fisher Scientific Co., Pittsburgh, Pa.) was added. The procedure, in general, was done according to Szybalski et al. (16). The SLS used here, in contrast to several other commercial products, was free from turbidity and color. The suspension, which lysed readily and became viscous but did not clear appreciably, was usually stored overnight at −40 C and after thawing was deproteinized by shaking with chloroform-butanol (3:1) for 5 min. It was then centrifuged for 10 min in a low-speed clinical centrifuge to break the emulsion, and the top aqueous layer was removed. This extraction was repeated until no protein film was evident. DNA was precipitated with 2 volumes of 95% ethyl alcohol, wound on a glass rod, rinsed in ethyl alcohol, and the glassy material was redissolved in either dilute SSC (1:10) or 0.02 M phosphate buffer, *p*H 7.2.

Modified SLS extraction. The above procedure was modified by the addition of one phenol extraction after the second chloroform-butanol step. Residual phenol was removed, and further deproteinization was carried out by one chloroform-butanol extraction. Effectiveness of this procedure in removing phenol was determined by extracting with uniformly labeled C^{14}-phenol diluted with stock phenol to a specific activity of 10 μc/ml. The extraction was then continued in the manner previously described. The final product, consisting of DNA which had been precipitated and redissolved in buffer, was assayed for radioactivity in a Packard Liquid Scintillation Counter. Representative samples gave only a background count and no quenching could be demonstrated, indicating complete removal of phenol.

Phenol extraction. Phenol (crystals, reagent grade; Merck & Co., Inc., Rahway, N.J.) was made 80% with distilled water. Samples were extracted at room temperature ("cold phenol") and at 50 C ("hot phenol") by use of modifications of the techniques of Weil (19) and Wecker (18), respectively. The suspensions were deproteinized by shaking for 5 min at the selected temperature with an equal volume of 80% phenol, centrifuged, and the aqueous layer and interface were removed. Extraction of the aqueous phase was repeated twice. After the final deproteinization, the aqueous portion was extracted three times with 2 volumes of ether in the cold to remove the phenol. Residual ether was removed by vacuum or by gently bubbling N_2 through the extract. DNA extracted by these methods was not precipitable upon addition of ethyl alcohol. Ether-free extracts were used for infectivity and other studies.

Modified phenol extraction. Samples were lysed with SLS, the suspensions were shaken with equal volumes of 80% phenol at room temperature, and the emulsions were separated as above. After three extractions of the aqueous layer, phenol was removed as above. The DNA precipitated by ethyl alcohol was granular and could not be wound on a rod, but it was easily collected by low-speed centrifugation and was readily soluble in either dilute SSC (1:10) or 0.02 M phosphate buffer, *p*H 7.2.

Spectrophotometry and determination of thermal transition curve. Spectrophotometric measurements were carried out where appropriate with a Beckman model B spectrophotometer and several ultraviolet spectrophotometers, i.e., Perkin-Elmer model 4000 automatic spectrophotometer, Perkin-Elmer (Hitachi) model 139, and a Gilford model 2000 multiple sample absorbance recorder with heating chamber and Haake circulating pump. The Gilford instrument was used to determine the melting-temperature curves of DNA. The temperature at the midpoint (T_m) of the increase in absorption was then determined. DNA used in these experiments was first dissolved in 0.1 M ethylenediaminetetraacetic acid (EDTA), *p*H 7.0, to prevent denaturation; then an appropriate amount of cold 8.0 M $NaClO_4$ was slowly added to a final molarity of 7.2 (3, 15*a*).

Analytical ultracentrifugation. Buoyant-density measurements of DNA in a CsCl gradient and calculation of GC content were carried out essentially as described by Schildkraut, Marmur, and Doty (14), with a Spinco model E ultracentrifuge equipped with ultraviolet optics and a four place AN-F rotor. Optical grade CsCl (The Harshaw Chemical Co., Cleveland, Ohio) was used; the optical density (OD) of a saturated solution was less than 0.05 at 260 mμ. Appropriate samples of extract containing 1 to 3 μg of DNA were mixed with concentrated CsCl, and SSC was added to a final density of 1.710 g/ml. The preparations were centrifuged at 25 C for 20 to 24 hr at 44,770 rev/min. Ultraviolet absorption photographs were taken on Kodak Commercial film. Preparations of DNA extracted (modified SLS) from *Micrococcus lysodeikticus* and *Escherichia coli* were employed as standards based on the values reported by Schildkraut et al. (14).

Electron microscopy. Purified fowlpox virus DNA was prepared and diluted in SSC to a final DNA concentration of 20 μg/ml. Freshly cleaved mica was coated with a thin layer of evaporated carbon. The mica surface then was scored with a sharp instrument to form squares approximately the size of electron microscope grids. The carbon was floated off the mica onto distilled water and picked up on polished copper grids, which were set aside to dry. One depression in a spot test plate was filled with an appropriate dilution of 0.265-μ polystyrene latex, and other depressions were filled with twofold serial dilutions of the DNA. The techniques used above have been described in detail (5). A carbon-coated grid was streaked across the surface of the polystyrene latex solution, and the small droplet adhering to the trailing edge was absorbed with bibulous paper. The grid was then streaked across the surface of one of the DNA dilutions, the adhering droplet was removed, and the grid was allowed to dry (4). Specimens were lightly shadowed with platinum at an angle of 7:1 in a Kinney model SC-3 vacuum evaporator. Grids were examined with a RCA electron microscope, model EMU-3G, equipped with a double condenser and a high magnification kit.

Isotopic labeling of viral DNA. Chicks, 1 day old, were infected with fowlpox virus and were given 15 μc of tritiated thymidine (H^3-TdR; specific activity, 1.9 c/mmole) intramuscularly at 3, 4, 5, and 6 days postinfection; the infected tissue was collected at 7 days. Virus was purified and the labeled DNA was prepared by the modified SLS extraction method. The radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Liquid Scintillation Counter.

Assay system for whole virus and DNA extracts. It has been repeatedly established that CAM and chick skin may be infected by whole virus with comparable efficiency. However, the infectious nature of extracted DNA could only be demonstrated by passage on the CAM followed by transfer of this material to the chick scalp, where lesions are specific and are easily read. The extracts were made 0.2 M with concentrated phosphate buffer, *p*H 7.2, before inoculation, and penicillin was added to give a concentration of 10,000 units per ml of extract. Each extract was tested on five or six embryonated eggs, 10 to 12 days old. The eggs were inoculated with 0.2 ml of extract, incubated for 5 days at 35 C, and the membranes were collected. Quantitation could not be attempted because of the difficulty of distinguishing infection from nonspecific lesions on the CAM. Microscopic examination of a suspicious CAM may show hyperplasia of ectoderm and an occasional inclusion. Other lesions may show only hyperplasia and may be nonspecific. Interpretation of CAM lesions was further complicated by the observation that some apparently normal membranes gave lesions on the scalp. The final and unequivocal test involves grinding of membranes, dilution in phosphate buffer, and brisk application of the resulting mixture onto freshly plucked 1-day-old chick scalps. These infected chick scalps, typical grossly and microscopically, yielded virus from which DNA could be extracted having the same physicochemical properties as that extracted from virus obtained by conventional methods (16).

RESULTS

Comparison of extraction procedures and infectivity of DNA. Ethyl alcohol precipitation of the DNA and solution in phosphate buffer or dilute SSC gave viscous solutions, with the exception of phenol-extracted material, as previously noted. Several methods of extraction are compared in Table 1. The phenol methods yielded the least (5 to 7%) DNA and SLS the most, approximately 80%. The latter method was modified because of considerable difficulty in dissolving ethyl alcohol precipitates. After the addition of one phenol step in the modified SLS method, the precipitates were readily solubilized, but the yield was less. A second extraction with phenol usually reduced the viscosity markedly, and very little or no DNA could be wound on a rod after precipitation with ethyl alcohol. The granular precipitate formed could be collected by light centrifugation.

TABLE 1. *Methods of DNA extraction**

Method	Yield†	Infectivity‡
	%	
"Hot" phenol, 50 C	5	4/4
"Cold" phenol, 25 C	7	2/4
Lysis with SLS, phenol (modified phenol)	38	4/6
SLS, chloroform-butanol	78	5/7
SLS (modified), chloroform-butanol, phenol, chloroform-butanol	68	10/13

* Details of the extraction procedures appear in Materials and Methods. The various reagents listed for a given procedure were used in the order given.

† Compared to diphenylamine reaction (Burton modification).

‡ Number of infectious extracts determined by inoculation onto CAM and subsequently onto chick scalps/total number of extracts. All data were collected over a period of 6 months.

It is of interest that another member of the poxvirus group, cowpox, resists phenol extraction. According to Joklik (7), no DNA was extracted by this method alone. Pfau and McCrea (10) reported that 10 to 15% of the total vaccinia DNA was released by phenol. In both of these excellent reports, DNA was extracted by other methods and was extensively characterized. DNA was not reported to be infectious in either of these studies.

The infectivity of the DNA preparations is shown in Table 1. All methods yielded infectious DNA However, these data require some explanation, as they may be misleading For reasons not known, the infectivity was variable. For several months, irrespective of the method of extraction, the infectivity of extracts was as high as 80%. At other times the DNA was not infectious, though the physicochemical properties were not significantly different.

It appears that the evidence for the infectivity of viral DNA rests primarily on qualitative observations. The amount of DNA seemed to bear no relationship to infectivity. The hot phenol method yielded little DNA, but it was the most infectious; however, even these preparations showed marked differences. For example, one extract containing 4 μg/ml gave a one-plus lesion (1 to 10 pocks) when titered by the dual assay system. A 1:10 dilution was negative. Another extract, containing 6 μg/ml, yielded a confluent infection. Similar data (without relationship to the amount of DNA) were obtained with material extracted by the other methods; all of these were more efficient in terms of yield. For instance, one

TABLE 2. *Assay of intact virus and DNA extracts*

Treatment	Infectivity	
	Extracts	Whole virus
Heat (100 C for 10 min)	+	0
Filtration (100 mμ)	+	0
Filtration (50 mμ)	+	0
Chloroform-butanol[a]	+	0
Deoxyribonuclease[b]	±	+
Trypsin[c]	+	+
Immune serum	+	—[d]

[a] Virus extracted twice, separated by centrifugation.

[b] Samples (1 ml) incubated at 37 C for 15 to 60 min; 0.005 M $MgSO_4$; 0.02 M phosphate buffer, *p*H 7.6; deoxyribonuclease, 100 to 200 μg/ml.

[c] Samples (1 ml) incubated at 37 C for 20 min; 0.02 M phosphate buffer, pH 7.6; trypsin, 100 μg/ml.

[d] 99% neutralized. Virus antiserum prepared in rabbits. Equal volumes (0.5 ml) of test material and serum incubated at 37 C for 2 hr, then titered. Titer reduced from 10^6 to 10^1.

extract of DNA (obtained by the modified SLS method) containing 133 μg/ml gave a confluent lesion, whereas another (110 μg/ml) gave a minimal lesion (10 to 25 pocks). Admittedly the dual assay system leaves a great deal to be desired as an accurate method of quantitation. Apparently, some factors of which we are not aware control infectivity.

The anomalous behavior manifested by the ability of the DNA to infect CAM only was perplexing. The fact that whole virus infected both CAM and chick skin with approximately equal efficiency tended to rule out virus contamination of the DNA. Furthermore, treatment of fowlpox virus with 2% SLS, or extraction with chloroform-butanol (followed by washing and collecting the residual virus pellet by centrifugation), effectively destroyed virus infectivity for chick skin.

These data notwithstanding, more rigorous proof that intact viral particles were not responsible for the infectivity of the DNA extract was desired. The data are presented in Table 2 and permit the reasonable conclusion that infectivity resides in the extracted DNA and not in contaminating whole virus. It is to be expected that fowlpox virus with an average size of 260 by 340 mμ (12) would be retained by filters 100 mμ and less in size. Heating also inactivated whole virus, but extracts were heat-stable and infectious for CAM. This characteristic has been demonstrated for DNA of other viruses (6, 20). The effect of deoxyribonuclease on infectivity of the extracts was not consistent. In some instances infectivity was entirely abolished; in others, some residual infectivity was preserved. The infective element was filterable through 50- and 10-mμ filters and was retained by dialysis. Deoxyribonuclease digest gave the characteristic increase in absorbance at 260 mμ (30%), and no banding was detected by CsCl gradient centrifugation. Furthermore, deoxyribonuclease treatment of H^3-labeled fowlpox virus DNA did not render the preparation entirely acid-soluble; 2% of the radioactivity was precipitable with cold perchloric acid after 15 min of digestion, and 1.3% after 60 min.

Some properties of extracted DNA. The DNA was extracted from inclusions and virus by the modified SLS method. The ultraviolet absorption curve was typical of DNA, as was the ratio of OD at 260 to 280 mμ (1.8 to 2.0). The protein content, as determined by the method of Lowry et al. (*7a*), varied from 0.5 to 3%, but was usually well below 3%. The buoyant density in CsCl (1.695 g/ml) was the same as that of native fowlpox virus DNA previously reported (16). Furthermore, only one band was ever seen by this technique which detects as little as 1 μg of DNA. Numerous preparations were examined in the electron microscope. Strands of material were abundant and easily recognized, with no viral particles evident in any of the preparations examined. The strands were destroyed when treated with deoxyribonuclease (100 μg/ml for 10 to 15 min at room temperature or 37 C).

Evidence of double-strandedness of DNA. The data just presented indicate that the extracted DNA was of good quality and possessed certain attributes associated with native double-stranded DNA. Several properties are now presented to show that fowlpox DNA is double-stranded.

Certain procedures affect double-stranded DNA with a consequent increase in absorption at 260 mμ (Table 3). Fowlpox virus, inclusion, and matrix DNA reacted slightly or not at all with HCHO at 37 C, even when held for prolonged periods. If single-stranded DNA were present, a continued increase in hyperchromicity with time of incubation would have been demonstrated (15). When samples were heated for 10 min at 100 C, the absorbance increased approximately 40% in the presence of HCHO, but only 12 to 16% without HCHO, indicating marked renaturation. Treatment with HCHO did not shift the absorption maximum from 260 mμ.

Treatment of extracts with deoxyribonuclease caused a significant and rapid increase in absorbance at 260 mμ, which averaged 30%. This behavior was characteristic of native double-stranded DNA (7, 15).

TABLE 3. *Effect of heating and formaldehyde on absorbance of DNA at 260 mμ**

Sample	Treatment	Temp	Time	OD at 260 mμ	Per cent increase
		C			
Virus DNA	Untreated			0.169	
	Heated	100	10 min	0.197	16.6
	HCHO, 1%	37	1 hr; 24 hr	0.180	6.5
		100	10 min	0.236	39.6
Matrix DNA	Untreated			0.299	
	Heated	100	10 min	0.337	12.7
	HCHO, 1%	37	1 hr; 24 hr	0.299	0
		100	10 min	0.415	38.8
Inclusion DNA	Untreated			0.358	
	Heated	100	10 min	0.403	12.5
	HCHO, 1%	37	1 hr; 24 hr	0.360	0
		100	10 min	0.508	41.9

* DNA was extracted by the modified SLS method, and samples were dissolved in SSC. After heating, the sealed ampoules were rapidly quenched at 0 C, and the OD at 260 mμ was determined in the Gilford spectrophotometer.

Other evidence used to establish the double-stranded nature of fowlpox virus DNA was supplied by the thermal transition curve, by use of the Gilford recording thermospectrophotometer. In 7.2 M $NaClO_4$ the T_m was determined to be 39 C, indicating a GC content of 34.6%, which confirmed the findings of Szybalski et al. (16). The increase in absorbance at 260 mμ was approximately 30%.

DISCUSSION

We have shown that DNA may be extracted from fowlpox virus and infected tissue by detergent and phenol methods, and that it is infectious for the CAM. The relative ease of extraction may be related to the high lipid content of fowlpox virus and matrix material (12). This might cause the virus to be more readily susceptible to the action of detergents by freeing the DNA without apparent excessive trauma to the molecule. Studies on the ultrastructure of the virus (5) reveal that short-term exposure of fowlpox virus to SLS uncoats the viral particles with ease, permitting extrusion of long strands of material susceptible to deoxyribonuclease. On the other hand, vaccinia virus (10), which contains much less lipid than fowlpox virus (21), and cowpox virus (7) require more rigorous extraction procedures to release the DNA.

It is of basic interest that the extracted DNA was infectious for the CAM but not for the chick skin, in spite of the fact that both of these tissues were infected by whole virus. It is interesting that the route of inoculation is highly critical also for foot-and-mouth disease viral nucleic acid (2). Ribonucleic acid obtained from this virus was infectious for mice by the intramuscular route but not intraperitoneally, whereas whole virus was highly infectious by both routes.

In the present study, the difference in tissue susceptibility to infectious DNA does not have a ready explanation. It is possible that the DNA molecule may not be able to penetrate the epithelial cells of the chick skin. The CAM, because it is an embryonic tissue, may have the requisite enzymes to initiate viral DNA synthesis; these enzymes might be lacking in the chick scalp.

It is possible that the extracted DNA molecule is in some way partially resistant to deoxyribonuclease because of the configuration of the molecule, a factor which may also explain the heat stability of the infectious DNA. Indeed, Szybalski et al. (16) have shown that approximately 15% of the DNA molecules of fowlpox virus have unusual properties of renaturation, suggesting that this may be due to cross-linking. These workers have suggested that such renaturation is a general phenomenon of animal virus DNA. In this respect, Russell and Crawford (13), Watson and Littlefield (17), and Weil (20) demonstrated the reversible thermal behavior of herpes, papilloma, and polyoma DNA, respectively. The findings of Weil (20) are particularly interesting in that both denatured and renatured polyoma virus DNA were infectious. Weil (19) also has shown that, after deoxyribonuclease treatment of polyoma subviral extracts (phenol), 1.6% of the control infectivity remained; Mandel (8) extracted poliovirus with SLS and found that about 5% of the infectious material (not related to whole virus) was ribonuclease-resistant. On the other hand, modification of the molecule or protection by histone or diamines (9) may be responsible for deoxyribonuclease resistance.

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