Intervirology *8:* 351–359 (1977)

Identification and Characterization of a Papillomavirus from Birds (Fringillidae)

ALBERT D. M. E. OSTERHAUS, DANIEL J. ELLENS and MARIAN C. HORZINEK

Institute of Virology, Utrecht State University, Utrecht,
and Central Veterinary Institute, Virology Department, Lelystad

Key Words. Avian papillomavirus · Purification · Electron microscopy · Structural proteins

Summary. From skin papillomas of the chaffinch *(Fringilla coelebs)*, a virus has been purified and studied by physicochemical techniques and electron microscopy. The virions measure 52 nm in diameter and are composed of 72 morphological units arranged in a skew T = 7d surface lattice. A sedimentation coefficient of about 300S and a buoyant density of 1.34 g/ml in CsCl were determined for the particle. Its protein composition resembles that of human papillomavirus, and the circular double-stranded genome measures 2.6 μm. This is the first demonstration of a member of the Papovaviridae family that affects a non-mammalian host.

The family Papovaviridae consists of two genera, *Papillomavirus* and *Polyomavirus* [1]. The most striking differences between members of these two genera are the size of the virions (55 and 45 nm in diameter, respectively), their sedimentation coefficients (296–300S and 240S, respectively), and their biological properties [2]. The genus *Papillomavirus* includes five types causing papillomas in man, rabbits, cows, dogs and hamsters.

Papillomaviruses had not been identified thus far in non-mammalian vertebrates. Warts have been reported in two closely related finch species, the chaffinch *(Fringilla coelebs)* and the brambling *(Fringilla montifringilla)*; the

Address inquiries to: ALBERT D. M. E. OSTERHAUS, Instituut voor Virologie, Faculteit der Diergeneeskunde, Rijksuniversiteit Utrecht, Yalelaan 1 – Praktikumgebouw, De Uithof, *Utrecht* (The Netherlands)

Received: December 23, 1976.

viral etiology was suggested from thin-section electron micrographs of papillomas which revealed the presence of intranuclear particles in paracrystalline arrays [3]. The present study reports the morphological and biophysical characterization of Fringilla papillomavirus (FPV), which must be considered the first avian member of the genus *Papillomavirus*.

Materials and Methods

Virus purification and isokinetic and isopycnic gradient centrifugation. FPV was purified from papillomas excised from the leg of a chaffinch *(Fringilla coelebs)*, kindly provided by Dr. P.H.C. LINA (Ministerie CRM, Rijswijk, The Netherlands). Human papillomavirus (HPV) was prepared from wart material removed surgically from patients, and kindly supplied by Dr. J. VAN BAAK (St. Anthonius Ziekenhuis, Utrecht, The Netherlands). After swelling in hypotonic buffer (phosphate-buffered saline containing 0.8 mM Na-EDTA, diluted 1:3 in aqua dest.), the warts were minced with scissors; the fragments were washed several times and subsequently ground in a mortar in 5 vol (w/v) of buffer. After blending in a Vortex mixer, the homogenate was frozen in a mortar, again ground thoroughly during thawing and left for 18 h at 4°. The suspension was clarified by low-speed centrifugation and the pellet was extracted once more. From the pooled supernatants the virus particles were pelleted at 50,000 rpm for 90 min in a Beckman SW50.1 rotor. Clarified 200-μl samples of the resuspended pellet were layered on top of isokinetic sucrose gradients [4] designed for particles with a density of 1.34 g/ml (sucrose top concentration 10% w/w). The gradients were spun at 5° in a Beckman SW50.1 rotor to reach a $\omega^2 t$ value of 4.03×10^{10} rad²/sec and were fractionated by top unloading with continuous OD_{280} recording. Pooled peak fractions (200 μl) were dialyzed and adjusted to a density of 1.34 g/ml with a Tris-HCl buffered solution of CsCl (pH 7.2). After centrifugation at 50,000 rpm for 24 h in a Beckman SW50.1 rotor, the gradients were fractionated and the densities of the peak fractions were calculated from their refractive indices [5, 6].

Electron microscopy. Fractionated material from isokinetic and isopycnic gradients was placed on carbon-coated copper grids. After 10 min of adsorption the grids were rinsed with 0.5 M ammonium acetate and were negatively stained for 10 sec using a 2% solution of potassium phosphotungstate, adjusted to pH 6.8 with KOH. The grids were inserted into the electron microscope (JEOL JEM 100 C) with the specimen side toward the electron source. For photographic enlargement the negative film was placed into the enlarger with the emulsion side away from the light source to allow correct reproduction of handedness. Instrumental magnification was calibrated by using a carbon replica of a diffraction grating (2,160 lines/mm; E. F. Fulham).

Extraction and spreading of DNA. DNA was phenol-extracted from a purified virus suspension according to PAGANO and HUTCHISON [7]. After precipitation with cold ethanol the DNA was dissolved in buffer (100 mM Tris-HCl, 10 mM Na_2-EDTA, pH 8.5) and spread under isodenaturing conditions onto a hypophase of 10% (v/v) formamide as described previously [8]. The grids were stained for 30 sec with 50 μM uranyl acetate in 90% ethanol and were rotary-shadowed with platinum-palladium (80–20). Length measurements were performed on micrographs using a map measurer.

Gel electrophoresis of iodinated viral polypeptides. For iodination of viral polypeptides, dialyzed (0.05 M sodium phosphate, 1 mM Na_2-EDTA, pH 7.5) 200-μl fractions from the isopycnic gradient were treated with 0.13 mCi of ^{125}I and 200 μg of chloramine-T [9]. After stopping the reaction with potassium bisulfite, the iodinated material was precipitated by incubation for 1 h at 4° in the presence of 6% trichloroacetic acid, bovine serum albumin and sodium deoxycholate [10]. For polypeptide analysis, the pelleted precipitate (7,000 rpm for 30 min, Beckman SW50.1 rotor) was washed twice with acetone and dried in a vacuum desiccator.

Polyacrylamide gel electrophoresis. The iodinated viral polypeptides and non-iodinated marker polypeptides were heated for 2 min at 100° in 0.075 M Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.003% bromophenol blue and 15% 2-mercaptoethanol. Samples were analyzed on slab gels [11] using the STUDIER [12] buffer systems. After electrophoresis at 100 V for 3 h, the gels were stained with 1% Coomassie blue in acetic acid-water-methanol (7:43:50%, by vol.). Gels were destained, dried in a vacuum on Whatman 3 MM filter paper, and autoradiographed on RP Royal X-omat film (Kodak, Rochester, N.Y.). Stained gels and autoradiographs were scanned densitometrically using a DD2 densitometer (Kipp & Zn., Delft, The Netherlands).

Results

Virus purification. The OD_{280} profiles from parallel isokinetic sucrose gradients of FPV and HPV extracts show distinct peaks at about 300S (fig. 1). The results of a centrifugation to isodensity in CsCl of peak material from the isokinetic run of FPV are demonstrated in figure 2. The peak in the extinction profile is situated at a density of 1.34 g/ml; when examined by electron microscopy, spherical particles of homogeneous size (fig. 3) were found accumulating in the OD_{280} peak fractions of both gradients.

Electron microscopy of FPV. The light-scattering gradient bands contained numerous full and empty virus particles. After negative staining with phosphotungstic acid these particles measured 51.7 ± 1.7 nm (n = 39) in diameter. A distinct pattern of morphological subunits could be recognized on the surface of the virion; their diameter was 7.6 ± 0.3 nm (n = 52). These subunits have also been observed as 'puddles' of capsomers in disintegrated virions (fig. 3). To determine the arrangement of the morphological subunits, one-sided particle images were analyzed (fig. 4). In all 27 particles examined, the path between neighboring 5-coordinated morphological units was two steps out and one to the right (5–6–6–5). Assuming that the side of the virion nearest to the carbon film was stained, we concluded that the morphological units of FPV are arranged according to a skew T = 7d icosahedral surface lattice; the same arrangement has been found for HPV [13] and could be confirmed in our studies (results not shown). Virions viewed down a 2-fold

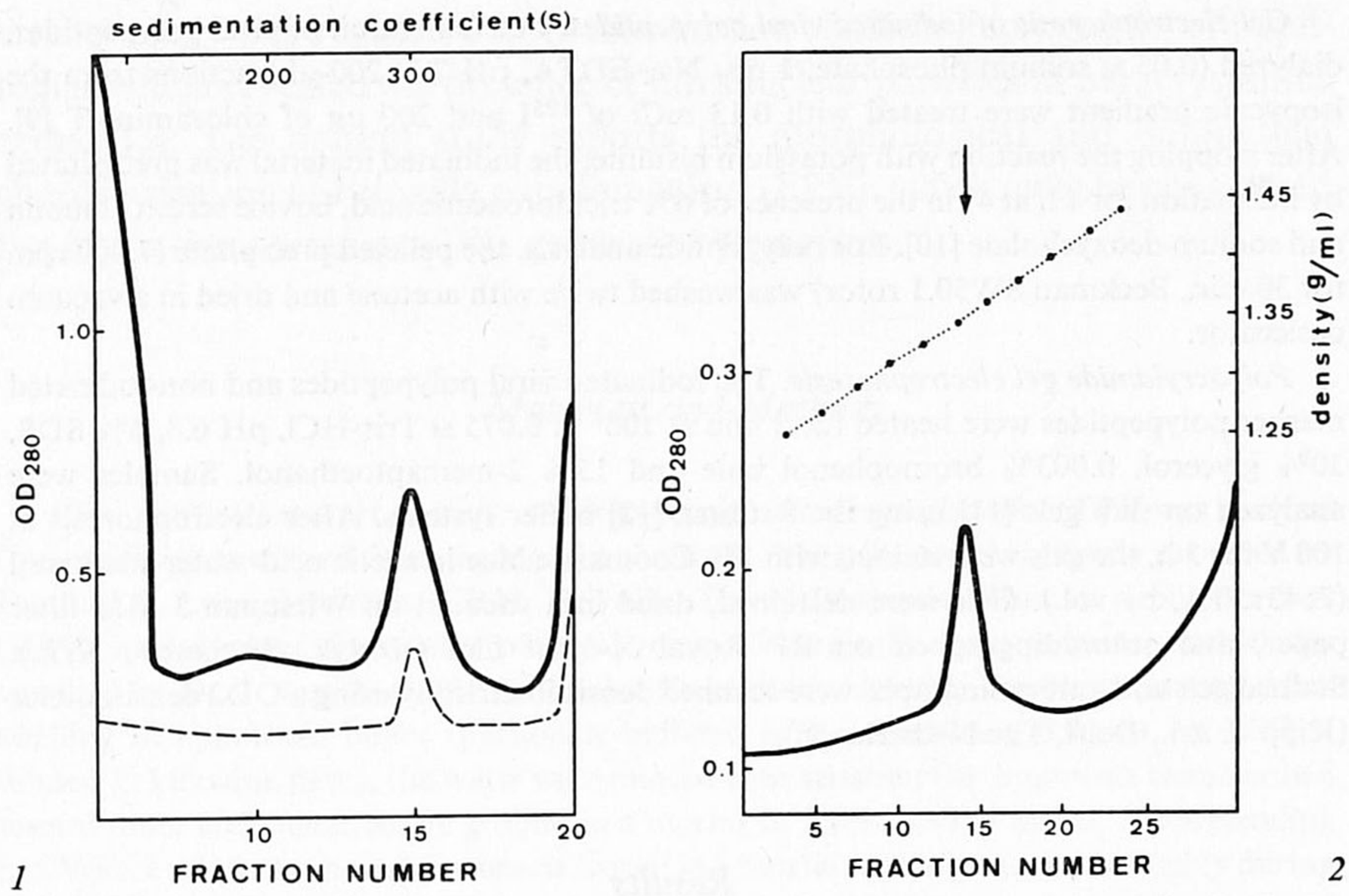

Fig. 1. OD_{280} profiles from parallel isokinetic sucrose gradients of FPV (——) and HPV (– – –) extracts.

Fig. 2. OD_{280} profile from isodensity centrifugation in CsCl of FPV peak material from the isokinetic run.

(fig. 4a), 3-fold (fig. 4b) or 5-fold axis (fig. 4c) can be recognized. The rotation technique of MARKHAM *et al.* [14] was used to accentuate the positioning of morphological units related to the axis of symmetry (fig. 4, bottom).

Comparative polypeptide analysis. Densitometric recordings from electropherograms of disintegrated and iodinated FPV and HPV gradient fractions run in parallel are shown in figure 6. Two large (mol. wt. >45,000), one middle-sized (mol. wt. 25,000–45,000), and two small (mol. wt. <25,000) proteins are prominent in both preparations; five additional minor peaks are found coinciding in both densitometric tracks (table I). Indications for the specificity of the polypeptide species listed have been obtained from a comparison of their relative proportions in different preparations, in adjacent gradient fractions and/or at varying times of autoradiographic exposure (results not shown).

Nucleic acid. Spreading of the nucleic acid extracted from purified FPV showed circular threads 2.6 ± 0.1 μm long (n = 15), sometimes displaying a superhelical configuration as has been demonstrated for the genomes of other papovaviruses (fig. 5) [15].

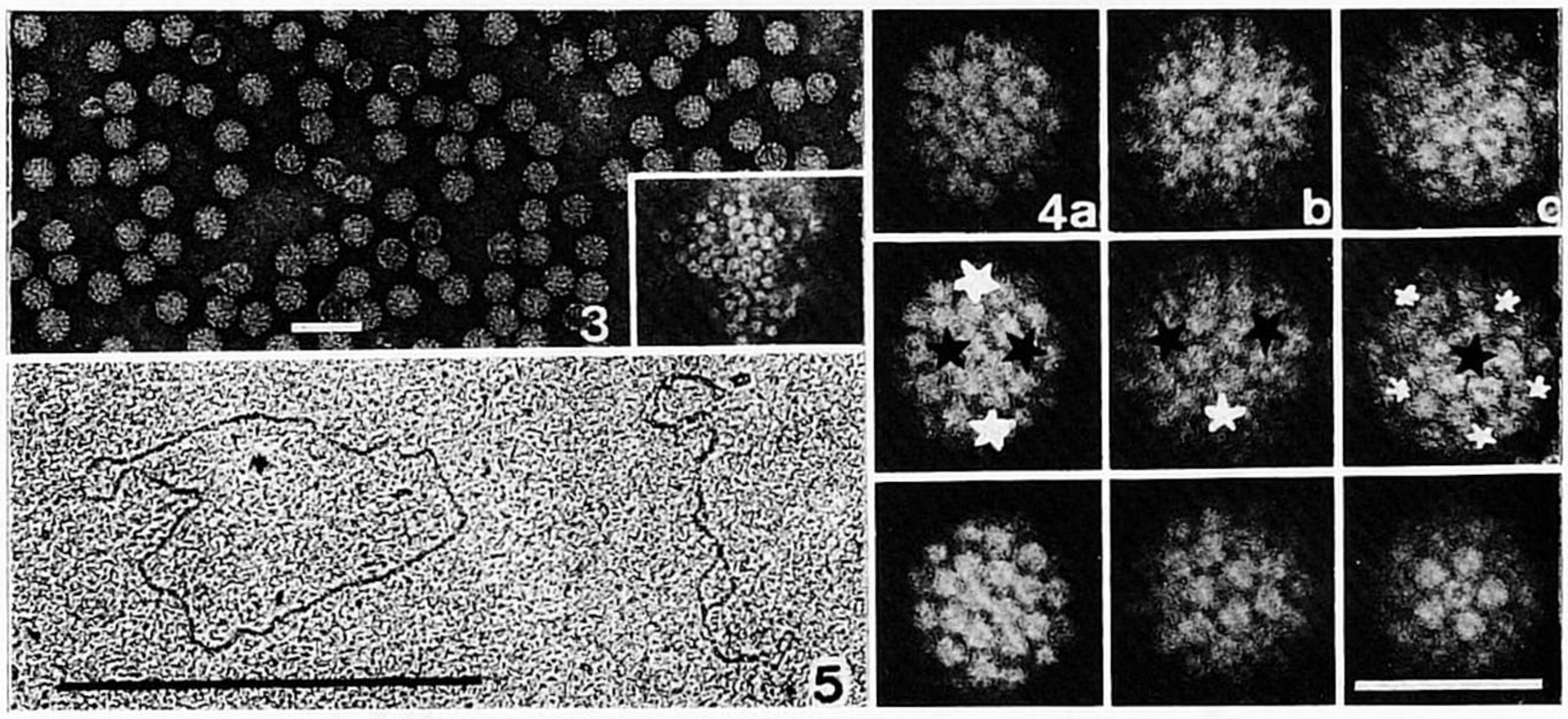

Fig. 3. Electron micrograph of 300S material from the isokinetic sucrose gradient depicted in figure 1. Bar = 100 nm. Inset shows a puddle of capsomers. × 112,000.

Fig. 4. a, b, c Avian papillomaviruses viewed down a 2-, 3- and 5-fold axis of symmetry, respectively. *Top:* Original electron micrographs. *Middle:* The 5-coordinated morphological units are marked with a star; the 5 points are directed toward the neighboring 6-coordinated morphological units. *Bottom:* Appearance of the particles after n = 2-, 3- and 5-fold optical rotation. Bar = 50 nm.

Fig. 5. DNA molecules extracted from the avian papillomavirus. The molecule on the right hand side is in the form of a supercoiled ring. Bar = 1 μm.

Table I. Molecular weights (× 10^{-3}) of FPV and HPV polypeptides[1]

FPV	HPV	FPV	HPV
>94	>94	32	32
83	83	17	18
66	68	12	12
59	61	<11	<11

[1] Determined by electrophoresis in a polyacrylamide slab gel (average from three determinations), using 2 μg of the following marker proteins: phosphorylase A (mol. wt. 94,000); bovine serum albumin (68,000); catalase (60,000); glutamate dehydrogenase (53,000); ovalbumin (43,000); aldolase (40,000); bovine lens protein (20,000); and cytochrome C (11,000).

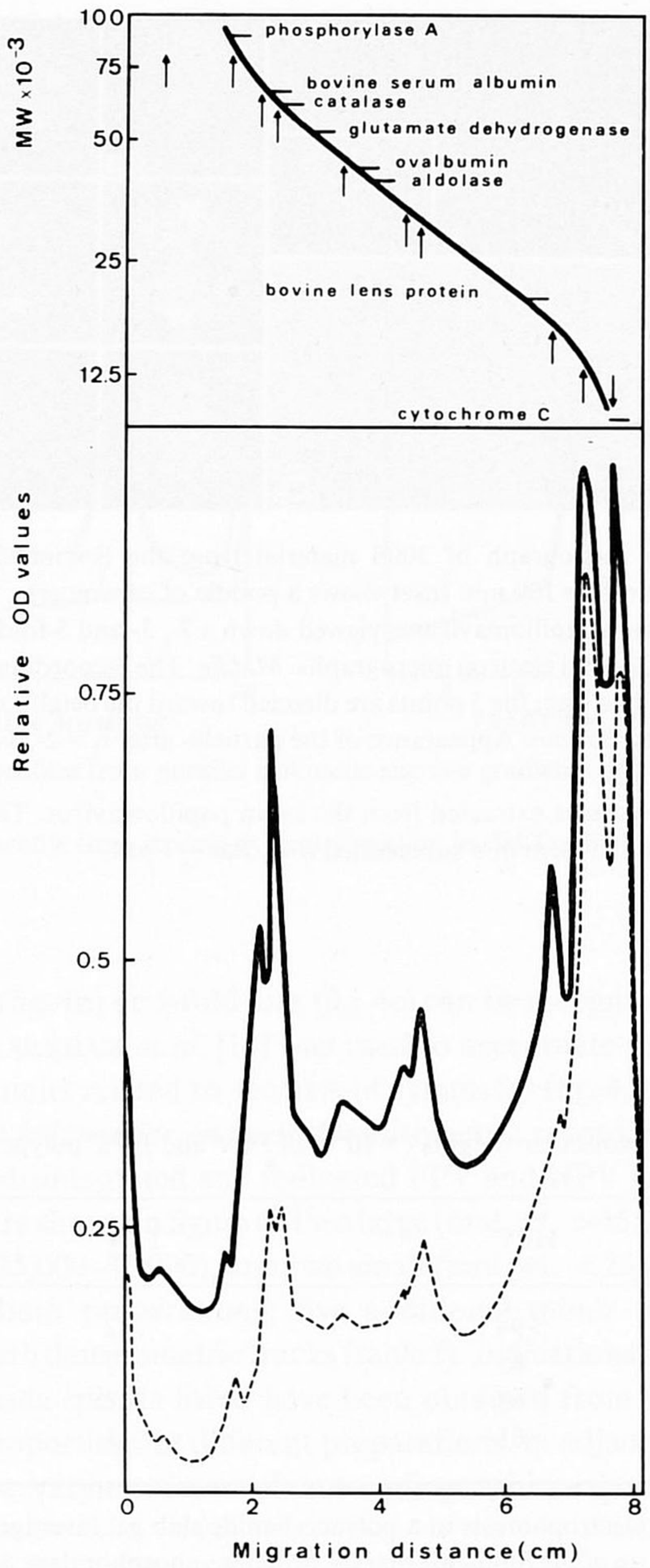

Fig. 6. Densitometric recordings from electropherograms of disintegrated and iodinated FPV (——) and HPV (– – –), run in parallel. Bars = Marker proteins (table I); arrows = polypeptides.

Table II. Comparison of chemical, physical, morphological and biological properties of FPV and other papillomaviruses

Property	FPV	Papillomaviruses[1]
Genome	circular double-stranded DNA length 2.6 μm	circular double-stranded DNA length 2.56 μm
Proteins	n = 10	n = 10
Density, g/ml	1.34	1.29–1.34
Sedimentation coefficient	300S	296–300S
Morphology	52 nm icosahedral 72 capsomers T = 7d	55 nm icosahedral 72 capsomers T = 7d or T = 7l
Natural host range	Fringilla species	respective host species
Association with disease	papillomas	papillomas

[1] Data taken from MELNICK *et al.* [2] and our own results with HPV.

Discussion

Electron microscopy of FPV revealed naked 52-nm particles with 72 morphological capsid subunits arranged in a skew T = 7d icosahedral surface lattice which is in accordance with the lattice of HPV [13]. For determination of the handedness of the lattice, it is essential to know which side of the virion has been contrasted. From stereomicrographs we concluded that the side nearest the carbon film was stained. Molecular weight determinations of FPV and HPV proteins by PAGE show very similar values for both viruses, although their relative proportions varied considerably (fig. 6). The congruity in the molecular weight distribution is taken as evidence supporting the viral specificity of these polypeptides. For viruses of hosts as distantly related by phylogeny as mammals and birds, this finding was unexpected in view of the lack of serologic [16] and genetic [15] relationships between other papillomaviruses. The observation that the genome of FPV is a circular molecule with the characteristics of a covalently closed double-stranded DNA is in accordance with results obtained for other papillomaviruses [15]. Its classification as a papillomavirus is based on its physical, morphological, chemical and biological properties, which resemble those of HPV (table II). Size, sedimentation coefficient and biological properties of the virions justify classification as a member of the genus *Papillomavirus*. Until now, members

of this genus were restricted to mammals, whereas FPV was purified from skin papillomas of a bird. In spite of intensive search in many wild and domestic bird species, these neoplasms could only be found in two closely related finch species, the chaffinch *(Fringilla coelebs)* and the brambling *(Fringilla montifringilla)* [P.C. LINA, personal commun.]. This may indicate a narrow host range, which is a common feature for papillomaviruses [2].

Acknowledgments

The authors wish to thank Miss A. KROON, Mr. P. DE KREEK and Mr. A. TIMMER for their skillful technical assistance, Mr. J. LOKKER for photography, and Miss J. VAN DEN HEILIGENBERG for preparation of the manuscript.

References

1 FENNER, F.: Classification and nomenclature of viruses. Intervirology *7:* 1–116 (1976).
2 MELNICK, J.L.; ALLISON, A.C.; BUTEL, J.S.; ECKHART, W.; EDDY, B.E.; KIT, S.; LEVINE, A.J.; MILES, J.A.R.; PAGANO, J.S.; SACHS, L., and VONKA, V.: Papovaviridae. Intervirology *3:* 106–120 (1974).
3 LINA, P.H.C.; VAN NOORD, M.J., and DE GROOT, F.G.: Detection of virus in squamous papillomas of the bird species *Fringilla coelebs*. J.natn.Cancer Inst. *50:* 567–571 (1973).
4 VAN DER ZEIJST, B.A.M. and BLOEMERS, H.P.J.: 4698 Isokinetic glycerol and sucrose gradients for density centrifugation; in TASMAN Handbook of biochemistry and molecular biology; 3rd ed., pp.426–520 (The Chemical Rubber Co., Cleveland 1976).
5 SOBER, H.A. (ed.): Handbook of biochemistry. Selected data for molecular biology; 2nd ed., pp. J292–J296 (The Chemical Rubber Co., Cleveland 1970).
6 DAWSON, R.M.C.; ELLIOT, D.C.; ELLIOT, W.H., and JONES, K.M. (eds.): Data for biochemical research, p.631 (Oxford University Press, London 1969).
7 PAGANO, J.S. and HUTCHISON, C.E.: Small, circular, viral DNA: preparation and analysis of SV40 and ϕX174 DNA; in MARAMOROSCH and KOPROWSKI Methods in virology, vol.5, pp.79–123 (Academic Press, New York 1971).
8 ELLENS, D.J.; SUSSENBACH, J.S., and JANSZ, H.S.: Studies on the mechanism of replication of adenovirus DNA. III. Electron microscopy of replicating DNA. Virology *61:* 427–442 (1974).
9 GREENWOOD, F.S.; HUNTER, W.M., and GLOVER, J.S.: The preparation of ^{131}I labelled human growth hormone of high specific radioactivity. Biochem.J. *89:* 114–123 (1963).
10 BENSADOUIN, A. and WEINSTEIN, D.: Assay for proteins in the presence of interfering materials. Analyt. Biochem. *70:* 251–257 (1976).
11 LAEMMLI, U.K.: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, Lond. *227:* 680–685 (1970).
12 STUDIER, F.W.: Analysis of bacteriophage T7 early RNA's and proteins on slab gels. J. molec. Biol. *79:* 237–248 (1973).

13 KLUG, A. and FINCH, J. T.: Structure of viruses of the papilloma-polyoma type. I. Human wart virus. J. molec. Biol. *11:* 403–423 (1965).
14 MARKHAM, R.; FREY, S., and HILLS, G. J.: Method for the enhancement of image detail and accentuation of structure in electron microscopy. Virology *20:* 88–102 (1963).
15 CRAWFORD, L. V.: Nucleic acids of tumor viruses. Adv. Virus Res. *14:* 89–153 (1969).
16 LE BOUVIER, G. L.; SUSSMAN, M., and CRAWFORD, L. V.: Antigenic diversity of mammalian papillomaviruses. J. gen. Microbiol. *45:* 497–501 (1966).