JOURNAL OF VIROLOGY, Mar. 1970, p. 381-387

Vol. 5, No. 3
Printed in U.S.A.

Chemical Studies on Polyoma and Shope Papilloma Viruses

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Received for publication 9 January 1970

Polyoma and Shope papilloma viruses were purified and analyzed by chemical and physical methods. Disc electrophoresis of degraded virions indicated the presence, in both cases, of only one major species of polypeptide subunit. The weight of the peptide chain of polyoma virus was estimated in 8 M urea to be about 45,000 avograms, based on the sedimentation rate in a sucrose-urea gradient and the diffusion coefficient estimated from the differential migration in electrophoresis in gels of different pore size. The presence of a minor peptide of smaller size was suggested by carboxyl-terminal and sedimentation analyses. The amino acid composition of polyoma capsid protein was reported. Chemical analyses showed that polyoma virus and Shope papilloma virus contained 16 and 17.5% deoxyribonucleic acid, respectively. Light scattering by the polyoma virion showed it to have a molecular weight of 22×10^6 and a diameter of 54 nm.

Polyoma and Shope papilloma viruses belong to a group of tumor viruses of similar electron microscopic appearance whose deoxyribonucleic acid (DNA) exists as a supercoiled circle. Purified preparations have shown the two viruses to differ slightly in the size of the virion and the size and content of the DNA (6, 14, 15, 31). End-group analysis of Shope papilloma virus suggested that only one type of peptide is present (14). Thorne and Warden (28) found only one electrophoretic peptide component in polyoma virions. Fine et al. (11) found the polypeptide of polyoma virus to have a molecular weight of about 50,000 and reported a minor peptide component of smaller size.

In this report, peptide analysis by electrophoresis in polyacrylamide gels showed the need for improved purification. More highly purified preparations of virions were analyzed for peptides, amino acid composition, and DNA content. A higher content of DNA and simpler peptide composition were found with both viruses. Polyoma virions were further studied for the size of the virion and the size of the peptide subunit.

MATERIALS AND METHODS

Virus production and purification. The large plaque (IL 11) strain of polyoma virus was grown and purified by the method of Winocour (31). Disc electrophoresis of the proteins of this preparation yielded a complex and variable pattern. Therefore, the purification procedure was modified to include enzymatic digestion, sonic treatment, and rate zonal centrifugation as follows. The cell lysate was incubated with 10 to 20 μg of pancreatic deoxyribonuclease (Worthington Biochemical Corp., crystalline) per ml at room temperature for 1 to 2 hr, followed by 100 μg of trypsin (Nutritional Biochemicals Corp.) per ml for 16 hr at room temperature. The high-speed pellet was suspended in CsCl (Harshaw Chemical Co.; density, 1.31 g/ml) buffered with 0.01 M tris (hydroxymethyl)aminomethane (Tris; *p*H 8.9), sonic treated for 3 min at full power in a Raytheon 10-kc sonic oscillator, and centrifuged in an SW 39 swinging bucket rotor. The visible band was dialyzed against Tris-buffered saline [0.15 M NaCl, 0.01 M Tris, *p*H 8.9 (TBS)], layered onto a 5 to 20% sucrose gradient, and centrifuged at 20 to 22 C for 25 min at 30,000 rev/min in an SW 39 rotor or for 45 min at 25,000 rev/min in an SW 25 rotor. To the visible band which had sedimented approximately 17 mm was added 0.39 g of CsCl per ml, and the preparation was centrifuged to equilibrium and dialyzed to TBS.

Shope papilloma virus was purified from cottontail rabbit warts (a gift of C. A. Knight) by the method of Crawford and Crawford (6). A part of the preparation was further purified by sonic treatment and centrifugation in a sucrose gradient.

Radioactive polyoma virus was produced by infecting 72-hr cultures of mouse kidney cells with 100 plaque-forming units (PFU) of polyoma virus per cell in phosphate-buffered saline. After a 2-hr adsorption period, the cells were washed with Eagle's medium plus 5% calf serum. Growth was continued in the same medium made without valine and leucine, to

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which was added 1 μc of ^{14}C-valine (specific activity 160 mc/mmole) and 10 μc of leucine-*4,5-*^{3}H (specific activity 7,600 mc/mmole) per 5-cm plastic petri dish. The yield of virus was only about 20% that of controls grown with complete medium, but the yield was not increased by enriching the depleted medium with 10% of the usual valine and leucine.

Biological assay of viruses. Polyoma virus was assayed by both its hemagglutination titer with guinea pig red blood cells and by its plaque-forming ability on monolayer cultures of primary mouse embryo cells (31).

Shope papilloma virus was assayed by its ability to produce warts on the shaven and scarified skin of domestic rabbits (14).

Light scattering measurements. The size of polyoma virus freshly isolated from a sucrose gradient dialyzed against TBS was determined by light scattering at 436 nm in a Sofica instrument. The solution was filtered directly into the cell through a 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) which had been previously treated with bovine serum albumin and washed to prevent adsorption of virus to the membrane (9). The concentration of virus was measured immediately after the light-scattering measurements. The refractive index increment was assumed to be 0.185 ± 0.005 cc/g (10).

Degradation of virus. The purified viruses were reduced and carboxymethylated by minor modifications of published procedures (7, 26).

Disc electrophoresis. Electrophoresis was performed in 7.5% polyacrylamide gels in glass tubing (inside diameter, 5 mm) at room temperature, with a current of 5 ma per tube. Discontinuous buffer and gel systems were used, with separation taking place either at *p*H 8.9 (8) or *p*H 4.3 (24). To prevent precipitation of the peptide chains derived from degraded viruses, all gels were polymerized in 8 M urea (recrystallized deionized urea was added to concentrated monomer solutions) or in 0.1% sodium dodecyl sulfate (SDS). With SDS electrophoresis, the detergent was present in the gels and electrode tanks, but with 8 M urea only the gels contained urea. To avoid exposure of proteins to decreased urea concentrations, an additional spacer gel containing 8 M urea was layered on top of the sample gel. Eastman chemicals were used to make the gels, except for the ammonium persulfate (Matheson Co., Inc.).

The gels were stained with amido black (8). For purposes of reproduction, the gels were photographed through an orange filter, and the negatives were scanned with a Joyce-Loebl microdensitometer at a slit width of 25 μm.

For the estimation of the diffusion coefficients (23), a smaller pore gel was made by increasing the acrylamide concentration to 15% and decreasing the methylene bisacrylamide to 0.1% (24). For some of the electrophoresis runs in the presence of SDS, which increased the electrophoretic mobilities, smaller pore gels (usually 13 and 20% acrylamide) were used to retard the peptides. When very dilute protein solutions were to be analyzed, the length of both the spacer and the sample gels was increased (23) to 3 cm.

Diffusion coefficients. The diffusion coefficient of the capsid protein of degraded polyoma virus was estimated by the method of Ornstein (23), which is based on the difference in frictional resistance in gels of different pore size. For calibration of the gels, the diffusion coefficients in 8 M urea of bovine serum albumin and ovalbumin were taken from McKenzie et al. (18). The diffusion coefficient of soy bean trypsin inhibitor (Worthington Biochemical Corp.) was measured in a Beckman apparatus by diffusion at 20 C of a 0.6% solution of protein in 8 M urea-TBS (0.2 M NaCl) against equilibrated buffer.

Sedimentation of capsid peptides. Degraded radioactive polyoma virus was analyzed by sedimentation in linear 5 to 20% sucrose gradients in 8 M urea-TBS. The sedimentation rate was estimated by comparison to bovine serum albumin and ribonuclease (20).

Measurement of phosphorus. Phosphorus was estimated by Bartlett's (2) sensitive modification of the Fiske-SubbaRow method. With a final volume of 1 ml, the sensitivity was such that 0.0039 μmole of P (equivalent to 1.3 μg of the sodium salt of DNA) produced absorbancy $(A)_{260}$ = 0.100. Phosphate was liberated from dried virus or DNA by heating with 0.1 ml of 70% perchloric acid (Fluka, Buchs, Switzerland) at 160 to 180 C in a sand bath for 8 hr.

Amino acid analysis and protein concentration. Amino acid analyses were performed on a Beckman Spinco amino acid analyzer, by using the high-speed and high-sensitivity systems. Protein split from the virus with phenol (25) was used for the amino acid analyses reported here. Some preparations of intact virus were analyzed for both phosphorus (*see above*) and amino acids. The content of DNA was based on the weight of protein calculated from the sum of the amino acid residues. For routine protein measurements, the method of Lowry et al. (17) was used, with bovine serum albumin as a secondary standard.

Hydrazinolysis was performed as described earlier (14), by using Fluka analytical grade hydrazine. Analyses were made of both intact lyophilized virus and protein split from virus with phenol.

RESULTS

Purification and disc electrophoresis. Analysis by disc electrophoresis of the two viruses prepared by standard purification procedures and degraded as described above showed multiple peptide components (Fig. 1A, 2A, and 3A). However, after additional steps of purification, the preparations contained essentially only a single peptide component (Fig. 1B, 1C, 2B, 2C, and 3B).

Alternate conditions of electrophoresis supported this finding. Electrophoresis of polyoma virus was carried out at two different *p*H values (Fig. 1B and 1C). Shope papilloma virus was also analyzed at *p*H 8.9 by using 0.1% SDS in place of urea to prevent precipitation of peptides. Although the electrophoretic mobility of the peptides was much higher in the presence of SDS, the patterns of peptides confirmed the measure-

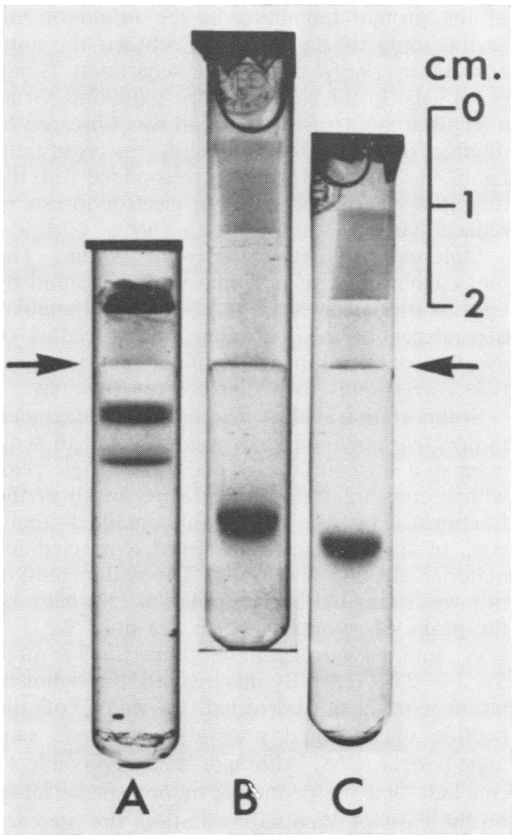

FIG. 1. *Electrophoretic pattern of protein subunits of polyoma virus in 8 M urea. Purified virus was degraded in 10 M urea, reduced, carboxymethylated, dialyzed against deionized 8 M urea, and run in disc electrophoresis. In (A) the virus was purified by differential centrifugation and equilibrium centrifugation in CsCl (31). Electrophoresis was at pH 4.3 for 1.5 hr. In (B) and (C), virus was further purified by enzyme treatment, rate zonal centrifugation in a sucrose gradient, and rebanding in CsCl. Electrophoresis was for 2.5 hr at pH 4.3 (B) or for 2 hr at pH 8.9 (C). The origin is at the top. The arrows indicate the effective origin of electrophoretic separation, where there is a change in the pH and the pore size of the gel. Since each preparation was electrophorated under different conditions, the distances traveled are not comparable.*

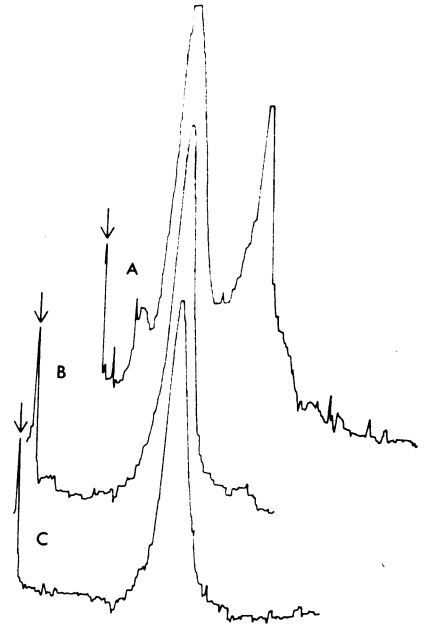

FIG. 2. *Densitometer tracings of the stained electrophorograms shown in Fig. 1. The arrows indicate the effective origin. The sample and spacer gels were somewhat turbid and were not included in the drawing.*

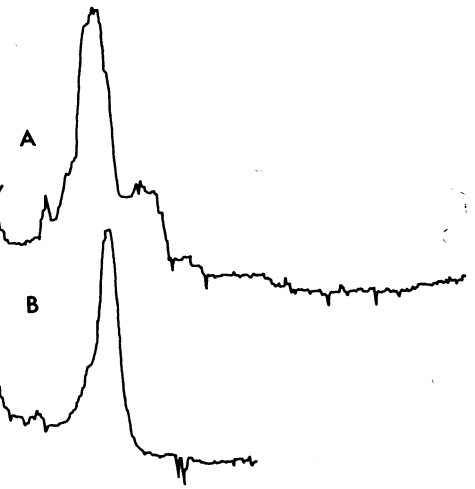

FIG. 3. *Densitometer trace of the electrophoretic pattern of protein subunits of Shope papilloma virus in 8 M urea. The virus was degraded as in Fig. 1, with electrophoresis at pH 4.3. In (A), the virus was purified by trypsinization, sonic treatment, differential centrifugation, and banding twice in CsCl. In (B), the same preparation was purified further by sonic treatment and rate zonal centrifugation. Since the latter preparation was considerably diluted, the anticonvection gels (sample and spacer gels) were sixfold longer than in the standard method.*

ments made in 8 M urea. In the presence of the detergent, a small amount of protein which always ran with the front was assumed to be a complex of protein and larger amounts of the ionic detergent (1).

Since recovery of material could not be tested by the staining technique, polyoma virus labeled with ^{14}C-valine and ^{3}H-leucine was purified and run in electrophoresis under the same conditions as in Fig. 1B. Thirty per cent of the applied

radioactivity remained in the anticonvection gel in which the sample was polymerized, 13% was trapped at the interface between the anticonvection and separation gels, and 48% was found in the stained band (Fig. 4). The ratio of isotopes in all fractions was not significantly different from the ratio in the purified virus ($^{14}C/^{3}H$ = 1.6).

Within the precision of the assays, the specific biological activities of the viruses were not changed during the more rigorous purification procedures. The discarding of 30 to 40% of extraneous protein indicated by the increase in DNA content (*see below*) and the removal of electrophoretic components would not be detectable in biological assays as performed here. The yield of polyoma virus in the last two steps of centrifugation as measured by A_{260} was 41%, with about half the loss in rate zonal centrifugation in a sucrose gradient and half in the equilibrium sedimentation in CsCl.

Treatment with mercaptoethanol was essential for the dissociation of both polyoma and Shope papilloma virus proteins. When either virus was degraded in 8 or 10 M urea, little or no material was observed to migrate into the gel, with most of the protein remaining at the origin or migrating only to the interface between the anticonvection (large pore) and separation (small pore) gels. In the case of Shope papilloma virus, a small amount of the material was observed to migrate into the separation gel in a series of faint bands, the fastest of which corresponded with the migrating species observed in electrophoresis of reduced virus.

Molecular weight of polyoma virion. The molecular weight of polyoma virus measured by light scattering was 22.3 × 10^6. The angular dependence of the scattering gave a radius of gyration of 21.3 nm, which for a spherical particle corresponds to a diameter of 54.4 nm.

Sedimentation studies. The protein of degraded radioactive polyoma virus was observed to sediment in a urea-sucrose gradient as a single peak with a constant ratio of ^{3}H to ^{14}C in all of the fractions (Fig. 5). The peak contained about 60% of the radioactivity applied, corrected for material striking the walls. The sedimentation rate was estimated by interpolation (20) between the peaks of ribonuclease (in 8 M urea $S_{20,w}$ = 1.5*S*) and bovine serum albumin [in 7 M urea $S_{20,w}$ = 2.4*S* (18)]. By this method, the sedimentation coefficient, corrected to water, of the peptide of degraded polyoma virus was estimated to be 1.6*S*. Although it is impossible to conclude that the peptide is monodisperse solely on the basis of zone sedimentation, the presence of a single symmetrical peak is at least consistent

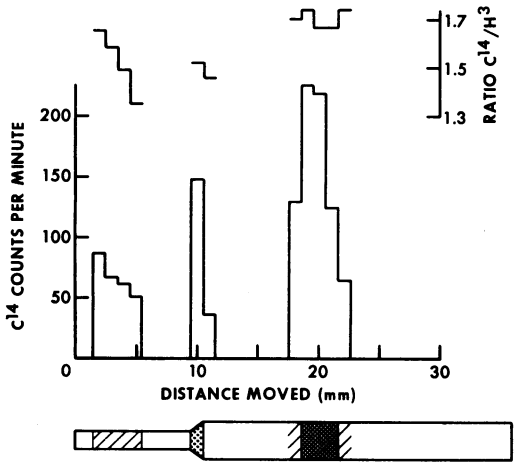

FIG. 4. *Distribution of radioactivity after disc electrophoresis of degraded polyoma virus labeled with ^{14}C-valine and ^{3}H-leucine. The virus was degraded by reduction and carboxymethylation in urea. Conditions of electrophoresis were as in Fig. 1C. The gels were stained to locate the visible bands for reference purposes, and the radioactivity was measured by slicing with a razor blade, dissolving the 1- to 2-mm slices in 0.5 ml of H_2O_2 at 37 C, and counting by liquid scintillation with the dioxane solvent of Bray (3). The ratios of isotopes are in the upper part of the figure, and a schematic scale drawing of the appearance of the stained gel is at the bottom of the figure.*

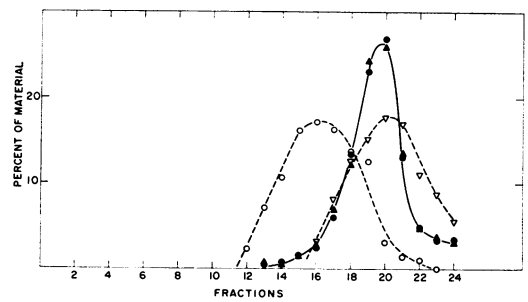

FIG. 5. *Sedimentation pattern of degraded polyoma virus labeled with ^{14}C-valine (●) and ^{3}H-leucine (▲). Purified virus was reduced and carboxymethylated in 8 M urea and mixed with bovine serum albumin and ribonuclease as markers. The mixture was centrifuged in a 5 to 20% sucrose gradient in 8 M urea at 18 to 20 C for 39 hr at 39,000 rev/min in an SW-39 swinging bucket rotor. The fractions were assayed for radioactivity by liquid scintillation in the solvent of Bray (3) with Cabosil added immediately. The bovine serum albumin was detected by A_{230} (○), and the ribonuclease was assayed enzymatically (▽) by the increase in acid-soluble nucleotides.*

with the existence of a single species. The constant ratio of isotopes across the peak provides further evidence on this point.

Diffusion coefficient of peptide. The diffusion coefficient of polyoma peptide was estimated by the method of Ornstein (23), based on the differential retardation of proteins during electrophoresis in gels of different pore size. In electrophoresis in 7.5 and 15% polyacrylamide gels containing 8 M urea, the peptide from degraded polyoma virus was observed (Fig. 6) to be retarded to almost the same extent as two of the standard proteins, bovine serum albumin and ovalbumin [in 7 M urea, $D_{20,w}$ = 3.1 and 3.2 Ficks, respectively (18)]. The diffusion coefficient of soy bean trypsin inhibitor in 8 M urea was found by classical techniques to be 9.5 Ficks, and, in agreement with the theory, it was much less retarded by gels of this pore size. Measurement of travel distances relative to the front (bromophenol blue marker) increased the reproducibility by minimizing any differences in current due to irregularities in the geometry of the apparatus.

Taking the diffusion coefficient of polyoma peptide in 8 M urea as 3.2 Ficks, corrected to 20 C in water, the molecular weight of the peptide can be calculated from the Svedberg equation. The partial specific volume can be calculated (5) from the amino acid composition (*see below*) assuming that only amino acids are present. In the Svedberg equation, $M = RTs/(1-\bar{v}\rho)D$, R = the gas constant, T = absolute temperature, s = sedimentation rate, $\bar{v}$ = partial specific volume, ρ = density of the solvent, and D = diffusion coefficient. Calculating from our data, $S_{20,w}$ = 1.6S, $D_{20,w}$ = 3.2 Ficks, and $\bar{v}$ = 0.73; the peptide molecular weight was 47,000. Marler et al. (19) found that in denaturing solvents such as guanidine hydrochloride, $\bar{v}$ is decreased by 0.02. Taking the value 0.71 for $\bar{v}$ would give a molecular weight of 43,000.

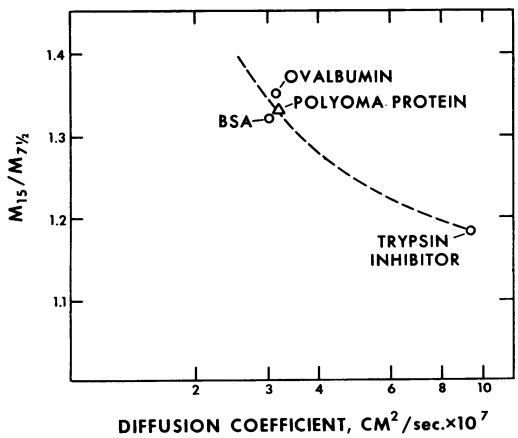

FIG. 6. *Estimation of diffusion coefficients from differential migration during electrophoresis in gels of different pore size. The electrophoretic mobility was measured in 8 M urea in 7.5% polyacrylamide gel and 15% polyacrylamide gel. For illustrative purposes, a line was drawn through the points representing the ratios of mobilities of known proteins in the different gels. From this data, only two points are known, so that the curvature of the line cannot be determined here (see reference 23).*

Chemical analysis. Chemical analyses performed on the purified viruses indicated that Shope papilloma virus contains a somewhat larger percentage of DNA than polyoma virus (Table 1). In both cases, the weight of protein was taken as the sum of the amino acid residues. The weight of the sodium salt of DNA was measured by phosphorus analysis and in some cases by diphenylamine as well. It was assumed that the virions contained only protein and DNA.

Amino acid analyses showed that polyoma virus contained all of the common amino acids (Table 2). Cysteine was not measured, but its presence was inferred from the requirement for mercaptoethanol to disaggregate the virus. Hare and Chan (13) reported similar evidence for disulfide bonds. Table 2 represents a composite of 24- and 72-hr hydrolyses analyzed by the high sensitivity method for both virions and protein recovered from phenol. The results were similar to those of Murakami et al. (21) for large plaque strains, except for a higher content of serine and alanine and a lower content of methionine. The amino acid analysis of a single preparation of Shope papilloma virus purified as in Fig. 3B was essentially the same as that reported earlier (14).

Preliminary results with hydrazinolysis of either intact polyoma virus or protein stripped by phenol treatment indicated serine to be the carboxyl-terminal amino acid, with an average

TABLE 1. *Deoxyribonucleic acid (DNA) content of polyoma and Shope papilloma viruses*

Virus	Protein concn[a]	DNA concn		DNA content[c]
		Diphenylamine[b]	Phosphorus[b]	
	mg/ml	*mg/ml*	*mg/ml*	%
Polyoma (11/65)...	0.55	0.10	0.103	15.8
Polyoma (4/66)....	0.84		0.160	16.0
Shope papilloma...	0.94	0.21	0.200	17.5

[a] Sum of amino acid residues.

[b] The sodium salt of DNA was taken as moles of deoxyribose × 660 or moles of P × 330.

[c] Calculated from phosphorus analyses.

TABLE 2. *Amino acid analysis[a] of polyoma virus*

Amino acid	Moles per 100 moles
Aspartic acid	9.5
Threonine	8.0
Serine	8.0
Glutamic acid	10.1
Proline	9.0
Glycine	8.7
Alanine	5.7
Valine	8.6
Methionine	1.3
Isoleucine	3.0
Leucine	8.6
Tyrosine	3.0
Phenylalanine	3.1
Lysine	6.5
Histidine	1.3
Ammonia	19[a]
Arginine	4.1
Tryptophan	2.0[b]

[a] Moles of ammonia not counted in total moles.
[b] Estimated by the method of Goodwin and Morton (12) from the ultraviolet spectrum in 0.1 N NaOH.

yield of 1 mole of serine per 92,000 g of protein. In view of the considerable losses encountered with this method (4, 22), this figure is compatible with the peptide chain weight arrived at by physical methods. Phenylalanine was recovered in amounts corresponding to 30 to 40% of the serine value, with a few other amino acids found in smaller quantities. In sedimentation studies (Fig. 5), the 5 to 8% of the radioactivity remaining at the meniscus was attributed to degraded material. In view of the phenylalanine found after hydrazinolysis, it cannot be ruled out that a small peptide component was present which was not detected in electrophoresis.

DISCUSSION

These studies indicate that a single type of peptide comprises the bulk, if not all, of the protein portion of the virions of strain IL 11 polyoma and Shope papilloma viruses. Alternate methods of analysis suggest a possible minor peptide component in polyoma virions. In view of the slow sedimenting component (Fig. 5) and the fairly substantial amounts of a second carboxyl-terminal amino acid, a small peptide may be present. This may be the material under study by Fine et al. (11) and Murakami et al. (21).

In searching for additional peptide components, the protein of polyoma virus was examined by electrophoresis at two different *p*H values, by sedimentation velocity, and by hydrazinolysis. The protein of Shope papilloma virus was examined by electrophoresis in both 8 M urea and 0.1% SDS. For Shope papilloma virus, the finding of a single peptide agrees with an earlier observation that threonine is the only carboxyl-terminal amino acid (14). However, at the present level of sensitivity, the possible presence of minor components cannot be ruled out. For example, the tracing in Fig. 2B suggested a minor component of higher mobility than the main component, but such a component was not seen in electrophoresis at the alternate *p*H (Fig. 2C). Similarly, the trailing edge of the peak in Fig. 3B indicated a possible minor component. In electrophoresis of radioactive polyoma peptide (Fig. 4), material which could contain additional peptides remained in the anticonvection gel and was thought to be aggregated or denatured, possibly by the vinyl polymerization. The DNA content of the two viruses as measured both by phosphorus and diphenylamine was higher than that found in previous studies (14, 21, 31). The nature of the adventitious peptide material removed by the enzymatic digestion, sonic treatment, and sucrose gradient centrifugation is not known, nor is it known at which step the peptides were removed.

Differences in amino acid composition between virions and empty capsids were used by Murakami et al. to show the presence of a small peptide in polyoma virions (21). In the present work, the small amount of material and the limited precision of amino acid analysis with the "high sensitivity" method made it impossible to detect differences between virions and phenol-prepared protein.

From the measurement of the weight of the polyoma virion by light scattering (22×10^6) and the chemical analysis of the DNA content (16%), the weight of polyoma virus DNA can be calculated to be 3.5×10^6. Measurements by electron microscopy and sedimentation analysis gave similar values (29, 30).

Taking the weight of the peptide chain of polyoma virus as 45,000, the virion would contain 410 repeating subunits. Klug (16) proposed a model of the polyoma virion which contains 420 ultimate structural units. Although the present findings are consistent with that model, it should be noted that the present methods for measuring the size of the peptide subunit are not highly accurate. In particular, the diffusion coefficient was estimated by a method which has not been previously used. In the measurement of sedimentation in urea, it was assumed that irregularities due to redistribution of urea in the centrifugal field were negligible. Furthermore, the measurement of both sedimentation and diffusion in concentrated urea solutions suffers from uncer-

tainties as to possible preferential solvation by the protein of either urea or water (27). Although the $S_{20,w}$ and $D_{20,w}$ were not intrinsic values, the measurements were made at low concentrations, so that errors due to concentration dependence should be minimal. In electrophoresis, protein concentrations of 25 to 100 μg/ml were applied, and in the sedimentation studies radioactive tracer quantities were used.

From these studies, it appears that about 25% of the genetic information of polyoma virus would be required to code for the peptide weighing 45,000 avograms, containing about 400 amino acid residues.

ACKNOWLEDGMENTS

The author thanks Leo Sachs, in whose laboratory the work was performed, and E. Winocour and P. Hausen for stimulating discussions and technical advice. I am grateful to Gerald Cohn for the light scattering measurements on polyoma virus and to Susan T. L. Kass for capable technical assistance.

This investigation was supported by grant PF-230 from the American Cancer Society.

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