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Synthesis and Assembly of Simian Virus 40

I. Differential Synthesis of Intact Virions and Empty Shells

HARVEY L. OZER

Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20014

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Intact virions and empty shells of simian virus 40 may be rapidly separated from each other and from cell contaminants by a procedure employing a CsCl cushion. This approach permits quantitation of their respective syntheses in infected cells labeled with radioactive amino acids. As much as 5 to 10% of the total acid-precipitable radioactive lysine in infected cell extracts was incorporated into viral particles in a two-hour pulse late in infection. Evidence for multiple origins of empty shells is presented. Some of the empty shells result from breakdown of intact virions. However, empty shells can also form independently of intact virions. First, labeling for periods of 15 min to 2 hr late in the course of infection results in preferential incorporation of ^{3}H-lysine into empty shells. Secondly, treatment with the deoxyribonucleic acid inhibitor cytosine-β-D-arabinofuranoside late in infection results in a 50% inhibition in the rate of formation of intact virions with minimal reduction in the rate of appearance of empty shells.

Little is known about synthesis of the viral structural proteins or assembly of the virion particle of the oncogenic deoxyribonucleic acid (DNA) virus, simian virus 40 (SV40). Electron microscopy has shown that viral particles are seen in the nucleus of permissively infected cells by 24 hr after infection. The particles appear in the cytoplasm only much later, at a time when degenerative morphological changes are occurring in the cells (8). Immunological analysis of infected cells has shown that an antigen or antigens (V antigen) can be demonstrated in the nucleus under conditions in which viral particles are present (17). Two main classes of viral particles can be purified from permissively infected cells: infectious, DNA-containing particles, density 1.33 g/cm^3 in CsCl ("intact virions") and noninfectious, DNA-free particles, density 1.30 g/cm^3 in CsCl ("empty shells"). Both react with anti-V serum (10, 11). The use of metabolic inhibitors has shown that the appearance of V antigen is prevented by inhibitors of protein or DNA synthesis (17).

The purified intact virions are composed of several polypeptide chains (1). Hitherto, it has not been possible to study their formation in infected cells above the high background level of persistent synthesis of cellular proteins. Similarly, no biochemical studies on the synthesis of viral particles have been reported.

To study the synthesis and assembly of SV40, a simple, rapid procedure which permits the isolation of viral particles from infected cells has been developed. The polypeptides in radioactively labeled virions and empty shells obtained by this procedure have been analyzed, and the syntheses of the two types of particles under different experimental conditions have been compared. In an accompanying paper (15), studies on the synthesis of the major structural protein of viral particles are reported. While this investigation was in progress, several workers reported on the composition of SV40 virions by sodium dodecyl sulfate (SDS)-acrylamide gel electrophoresis (3, 5, 7).

MATERIALS AND METHODS

Virus. The wild-type SV40 used was SV-S, isolated by Takemoto et al. (21). The several preparations of virus used during the experiments were all prepared by a single passage in Vero cells at a multiplicity of infection (MOI) of 0.01 to 0.1 from a common stock prepared in primary African green monkey kidney cells. Temperature-sensitive mutants, isolated in AH-1 cells (22), were passed once in Vero cells at low MOI. Virus pools were prepared by repeated freezing and thawing of cells at 3+/4+ (i.e., 75% cell lysis) cytopathic effect (CPE) in 50 ml of medium. The cellular debris was removed by low-speed centrifugation, and portions of the supernatant fluid were frozen at −20 C. Infectious units in the different virus pools were determined by the method of Robb and Martin (18; kindly performed by James Robb). Pool 14 contained approximately equal amounts of infectious virus and V antigen-inducing defective virions (24). All other

pools contained negligible amounts of defective virions. MOI values reported for this pool refer to total V antigen-inducing virus.

Cell line. Vero cells were obtained from American Type Culture Collection (CCL 81) and maintained in Eagle's no. 2 minimal essential medium supplemented with 2 × amino acids (complete medium) and 10% fetal calf serum (GIBCO). Glutamine was added immediately before use. Several tests for mycoplasma contamination of the Vero cells were negative during the course of the experiments.

Solutions. Unless otherwise noted, all solutions were in 0.01 M $NaH_2PO_4 \cdot Na_2HPO_4$, *p*H 7.2 (NaP).

Preparation of viral particle standards. Double-labeled SV40 was prepared from cells inoculated with an MOI of 0.5. At 2 days postinfection, ^{3}H-arginine (100 μCi/50 ml) and ^{14}C-valine (10 μCi/50 ml) were added. At 7 days postinfection (3+ CPE), the medium from several bottles was pooled and concentrated 10-fold overnight by dialysis against Aquacide (Calbiochem). Virus particles were isolated by velocity sedimentation into a CsCl cushion (1.40 g/cm^3) with subsequent equilibrium centrifugation (two cycles) of the separated viral particles in CsCl (1.34 g/cm^3). After dialysis and removal of precipitated material by low-speed centrifugation (2,000 rev/min for 10 min), the virus was further purified by sedimentation in a 5 to 20% sucrose gradient [0.1 M NaCl, 0.01 M ethylenediaminetetraacetic acid, 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.4] in an SW27 rotor at 23,000 rev/min for 90 min at 4 C. The peak tubes were pooled, dialyzed against NaP, and concentrated by pressure dialysis (collodion bag apparatus, Schleicher and Schuell).

Preparation of radioactive lysine-labeled infected cells. Confluent monolayers of Vero cells in 32-oz (0.946 liter) bottles (3 × 10^7 cells) were inoculated with 5 ml of stock virus for 2 hr at room temperature. After removal of the unabsorbed virus, 50 ml of complete medium with 1% fetal calf serum was added, and the cells were incubated at 37 to 38 C, unless otherwise noted. Prior to pulse-labeling, the medium was aspirated and the monolayer washed once with 25 ml of lysine-free complete medium. For short pulse (less than 3 hr), 20 ml of lysine-free medium with 1% fetal calf serum and 10 μCi of radioactive lysine per ml was added. For longer labeling periods, medium with a lysine concentration 10% of that in Eagle's medium and 1% fetal calf serum was used. After labeling periods, the cells were harvested in the labeling medium with a rubber policeman. The cells were sedimented at 2,000 rev/min (International model PR-6 centrifuge) for 10 min, the supernatant fluid was aspirated, and the cells were stored frozen at −20 C.

CsCl analysis of radioactively labeled infected cells. Frozen infected cells (0.1 to 0.15 ml packed volume) were thawed in 0.5 ml of NaP and disrupted by external sonic treatment for 5 min (Raytheon sonifier, 9 kc, 100 to 150 v output) at 4 to 8 C. The sonically treated material was centrifuged at 10,000 × *g* for 10 min at 4 C, and the turbid supernatant solution was removed (10,000 × *g* supernatant solution). The supernant solution was made 0.5 to 1% with the nonionic detergent, Nonidet P-40 (Shell), and incubated for 2 to 3 min at 4 C, The sample was placed onto a freshly prepared step gradient consisting of a layer of 2 to 2.5 ml of 15% sucrose in 0.15 M NaCl, NaP, over 2 ml of CsCl (range 1.32 to 1.33 g/cm^3 in NaP). The sample was centrifuged in an SW50.1 rotor at 40,000 rev/min for 80 min at 15 C and arrested without breaking. After centrifugation, two sharp bands were visible in the CsCl. The gradient was collected from the bottom in 10-drop fractions (0.14 ml per fraction). The appropriate two- to three-peak tubes were pooled and dialyzed overnight against 100 to 1,000 volumes of NaP for further analysis.

Several different concentrations of CsCl cushions were studied. Satisfactory resolution of highly purified, intact virions and empty shells were obtained only with a linear CsCl gradient or a CsCl cushion of 1.32 to 1.33 g/cm^3. The latter procedure was chosen for use with cell extracts because of its greater convenience.

Acrylamide gel electrophoresis. Ten per cent neutral acrylamide gels (5 cm) with 0.1% SDS, prepared by the method of Summers et al. (20), were used after overnight polymerization. Samples (0.05 to 0.1 ml) were treated with 1% SDS, 0.01 to 0.1 M dithiothreitol (DTT), NaP, at 37 C for 1 to 2 hr and at 100 C for 1 to 2 min. After addition of sucrose and marker dye (pyronin Y, 1 μliter of a 1 mg/ml solution), the samples were subjected to electrophoresis (Canalco) for 3.5 to 4 hr at 3.5 ma per tube at room temperature. In some cases, 5 μliters of purified, nonradioactive SV40 virions (2 mg/ml) was added to the sample as an internal marker. Gels containing radioactive proteins were frozen, sliced (1.3 mm per slice), and solubilized with 30% H_2O_2 by the method of Moss and Salzman (13). Samples were then counted in 1 ml of water and 10 ml of Triton-toluene (75% ± 5% recovery for ^{3}H, uncorrected for quench) or 1 ml of Nuclear-Chicago solubilizer and 10 ml of Liquifluor (95% ^{3}H recovery, 100% ^{14}C recovery). For all gels containing ^{3}H- and ^{14}C-protein, NCS-Liquifluor was used, and appropriate channel corrections were performed. The percentage of radioactivity in each peak was determined on the basis of the total radioactivity recovered.

Gels were stained in 0.05% Coomassie Blue in 10% trichloroacetic acid for 1 to 3 hr after overnight fixation in 20% trichloroacetic acid. For cases in which radioactivity on the gels was to be determined, the gels were briefly destained in 10% trichloroacetic acid, and the slices containing the stained marker, major capsid protein were analyzed as described above. The percentage of gel radioactivity in this capsid protein was calculated based on the radioactivity applied to the gel, assuming a recovery of 75% for the total gel. Total recovery of radioactivity after the staining procedure in several experiments was indistinguishable from the standard recovery in the absence of staining.

Determination of radioactivity. Radioactivity was determined by liquid scintillation spectrometry in a Packard model no. 3003 spectrometer. Aqueous samples were counted in Triton-toluene (17). Cell extracts (30 to 100 μg of protein) were precipitated in 7% trichloroacetic acid for 60 min at 4 C. Samples were collected on fiberglass filters (Whatman, GF/C) and dried. Precipitates were dissolved in 0.2 ml of Nuclear-

Chicago solubilizer at 50 C for 10 min, neutralized with 10 μliters of concentrated H_2SO_4, and counted in 10 ml of 1,3,4-phenylbiphenylyloxadiazole-toluene (Nuclear-Chicago Corp.).

Protein determination. Protein determinations were performed by the method of Lowry et al. (12), with bovine serum albumin (Armor Pharmaceutical Co.) as standard.

Serology. Viral particles (V antigen) were assayed by complement fixation using a guinea pig antivirus antiserum (kindly provided by David Hoggan) as previously reported (15). One complement fixation unit (CFU) in 25 μliters approximately equals 0.025 μg of purified viral particles.

Radioisotopes and chemicals. The following radioisotopes, obtained from Schwarz/Mann, Div. of Becton, Dickinson & Co., were used: ^{3}H-reconstituted protein hydrolysate, ^{3}H-lysine (6.5 Ci/mmole) ^{14}C-lysine (0.3 Ci/mmole), ^{3}H-arginine (18 Ci/mmole), ^{14}C-valine (0.25 Ci/mmole), thymidine-*methyl*-^{3}*H* (5 Ci/mmole) ^{14}C-thymidine (0.05 Ci/mmole), and ^{3}H-uridine (29.9 Ci/mmole). The following chemicals were used: CsCl (Schwarz BioResearch, Inc.; ultrapure, optical grade), sucrose (Mann, ultrapure), SDS (Mann. ultrapure), DTT (Calbiochem; "Cleland's reagent," A grade), and cytosine-β-D-arabinofuranoside-hydrochloride (AraC; Calbiochem, A grade).

RESULTS

Separation of viral particles on CsCl cushions. For an understanding of SV40 synthesis and assembly, it was necessary to clarify the physiological relationship between intact virions and empty shells. Three major possibilities were considered: (i) that empty shells were degradation products of intact virions; (ii) that empty shells were precursors of intact virions, as already suggested for poliovirus (9); and (iii) that the two particles were independently derived. A fundamental prerequisite to such a study would be a rapid separation of the two particles from intracellular material and from each other.

None of the described procedures for isolation of viral particles from cells satisfies this prerequisite. Extraction of intracellular virus by the method of Black et al. (4), Uchida et al. (24), or Koch et al. (11) involves prolonged treatment of disrupted cells before separation of particles. Variable quantities of the two particles were noted in those studies. Consequently, the following procedure was developed which appeared to obviate these difficulties.

Briefly, radioactively labeled, infected cells were harvested late in infection but before gross cytopathic changes (*see above*). The virus was extracted by sonic treatment, and the low-speed supernatant fluid was sedimented through sucrose into a CsCl "cushion," permitting resolution of the viral particles from each other and from the major cell components. Samples were briefly treated with NP-40 (0.5 to 1%) at 4 C immediately before analysis to diminish virus-trapping in membranous material which would accumulate at the CsCl-sucrose interface. Figure 1 shows typical patterns of ^{3}H-lysine-labeled, infected and uninfected cells from separate gradients. The two peaks were then analyzed either directly on SDS-acrylamide gels or after further purification by equilibrium centrifugation in CsCl The various aspects of the isolation and degree of purity or viral particles prepared on a CsCl cushion are detailed below.

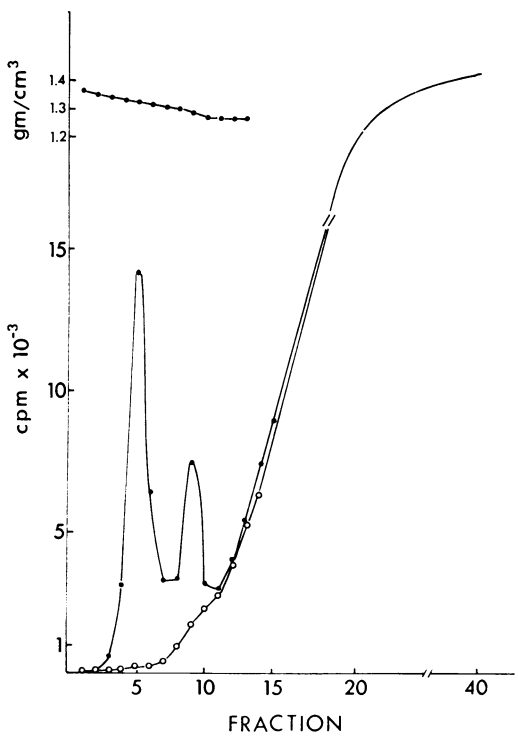

FIG. 1. *CsCl cushion analysis of infected and uninfected cells. Infected and uninfected cells were labeled at 44 to 60 hr postinfection with ^{3}H-lysine. After extraction by sonic treatment and centrifugation at 10,000 × g, the supernatant fluids were each analyzed on separate CsCl cushions (1.32 g/cm^3) as described in the text. The CsCl portion appears in fractions 1–12. Ten-microliter samples of each fraction were assayed for radioactivity in water-Triton-toluene. The trichloroacetic acid-precipitable radioactivity applied to CsCl 6.5 × 10^6 counts/min for infected cells (●—●) and 5.4 × 10^6 counts/min for uninfected cells (○—○).*

Identification of viral particles in CsCl cushion. The two radioactive-labeled peaks in the CsCl cushion were identified as intact virions and empty shells on the basis of cosedimentation with purified particles. The peak further into the cushion consisted of intact virions based on the following additional criteria. CsCl equilibrium analysis yielded a single peak at 1.33 g/cm^3 and electron microscopy showed greater than 90%

intact virions by phosphotungstic acid-negative staining. The second peak consisted of heterogeneous empty shells with few intact virions, as shown by electron microscropy, similar to that observed by Koch et al. (11). On CsCl equilibrium analysis, a major peak at 1.30 g/cm^3 was obtained.

Extraction of cells. Infected cells were frozen and stored at 20 C. The recovery of virus particles in the 10,000 × *g* cell supernatant fluid was determined directly or after various degrees of sonic treatment. The fractions of protein, trichloroacetic acid-precipitable radioactivity, and V antigen extracted are shown in Table 1. The relative yields of the two viral particles differed with the intensity of sonic treatment, both reaching a plateau at 750 volt-minutes (output volts times minutes of sonic treatment). Recovery of intact virions increased only slightly as a function of the duration of sonic treatment reaching a plateau at 1.5- to 2-fold that of the extract that was not sonically treated. On the other hand, there was a 16-fold increment in the quantity of empty shells obtained after sonic treatment. These results could represent either differential extraction of particles from cells or breakdown of intact virions into empty shells. To clarify these possibilities, reconstruction experiments were performed.

Effect of sonic treatment on viral particles. Reconstruction experiments (Table 2) were performed with the pooled intact virion or empty shell fractions described in Table 1. Unlabeled, infected cells (48 hr postinfection) were harvested and centrifuged. A 50-μliter amount of radioactive virus was added to the pelleted cells from one 32-oz bottle (0.1 ml of cells), and the mixture was frozen. After thawing, a sample was treated sonically for 10 min. Both sonically treated and untreated cell extracts were centrifuged at 10,000 × *g*, and the supernatant fluids were analyzed on a CsCl cushion as described above (extraction no. 1). The 10,000 × *g* pellets from both samples were suspended, pooled, frozen and thawed, and retreated sonically for 5 min. The 10,000 × *g* supernatant fractions from these recycled samples were also analyzed (extraction no. 2). The results are shown in Table 2. It can be seen that only 20% of the intact virions were detected in the region of empty shells of the CsCl cushion even after prolonged and repeated sonic treatment. In reconstruction experiments with empty shell material, the majority of recovered radioactivity was in the region of empty shells in the CsCl cushion with a leading edge in the region of intact virions. These results indicated that the counts in the region of empty shells were not predominantly attributable to breakdown of intact virions during the extraction procedure.

Nonviral particle radioactivity in CsCl cushions. Extracts of infected and uninfected cells, radiolabeled with thymidine or uridine, were analyzed after sonic treatment and NP-40 treatment. In all cases, radioactivity was observed in the CsCl cushion.

Thymidine radioactivity was distributed as a broad peak centered between the two viral particle peaks, whereas uridine radioactivity appeared as a narrow peak overlapping the region of empty shells. Treatment with deoxyribonuclease

TABLE 1. *Effect of sonic treatment on virion recovery*[a]

Intensity of sonic treatment (volt-minutes)	% Extracted[b]			CsCl cushion[c]			
				Counts/min		CFU	
	CFU	Protein	Counts/min	Intact virions	Empty shells	Intact virions	Empty shells
0	12	30	28	63,400	8,700	128	32
200	17	31	24	85,000	17,500	128	32
500	36	37	58	110,000	67,400	128	128
750	83	72	86	108,000	146,000	256	256
1,500	88	80	80	114,000	141,000	512	256

[a] Infected cells (MOI 70) were labeled with ^{3}H mixed amino acids in complete medium with 1% fetal calf serum from 48 to 68 hr postinfection (0.25 mCi per bottle). Portions of the pooled, freeze-thawed cell suspension (20% v/v) were treated sonically for 2 to 10 min at different intensities (intensity of sonic treatment is expressed in volt-minutes, which equal the minutes of treatment times the output volts). There were 5 × 10^6 trichloroacetic acid-precipitable counts per min per sample.

[b] Complement fixation units (CFU), protein, and counts per min in supernatant extract (10,000 × *g*) of sonically treated material expressed as per cent of total (supernatant fluid and pellet).

[c] Analysis of 10,000 × *g* supernatant extract was performed on CsCl cushion (1.33 g/cm^3) as described in Materials and Methods; counts/min for peak fractions are as shown in Fig. 3; CFU expressed as number of complement-fixation units of V antigen per 25 μliters after dialysis.

TABLE 2. *Reconstruction experiment*[a]

Treatment	Viral particles added[b]	Recovery in 10,000 × *g* supernatant fraction[c]		Recovery in CsCl cushion		% In empty shell peak[d]
		Counts/min	Protein (mg)	Intact virions (counts/min)	Empty shell (counts/min)	
None[e]	Intact virions	10,000		5,571	907	14
	Empty shells	8,500		365	5,130	
Extraction 1[f]						
No sonic treatment	Intact virions	1,573	2.8	683	73	10
	Empty shells	720	1.9	21	195	
Sonic treatment	Intact virions	3,780	3.6	2,517	457	15
	Empty shells	2,900	3.4	312	1,488	
Extraction 2[g]						
No sonic treatment	Intact virions	1,680		1,204	248	17
Sonic treatment	Intact virions	3,010		1,877	479	20

[a] A 0.1-ml amount of infected cells (48 hr postinfection, MOI 7) was mixed with 0.05 ml of ^{3}H mixed amino acid-labeled viral particles and frozen at −20 C. Cell-virus extracts were analyzed on CsCl gradients as described in the text.

[b] Pooled peaks of intact virions (10,000 counts/min) or empty shells (8,500 counts/min) from Table 1, dialyzed and stored for 2 weeks in $NaH_2PO_4 \cdot Na_2HPO_4$, *p*H 7.2 (NaP), at 4 C, were added per 0.1 ml of cells before freezing.

[c] Counts per minute or milligrams of protein in 10,000 × *g* supernatant fluid to be analyzed on CsCl. The protein extracted was 40% of the total (supernatant fluid plus pellet) for preparations which were not sonically treated and 58 to 63% for sonically treated preparations.

[d] Counts per minute in region of empty shells divided by counts per minute in region of intact virions and empty shells (× 100).

[e] Virus particles analyzed directly on CsCl without cells, prior freezing, or sonic treatment.

[f] Virus-cell mixture, thawed in 0.3 ml of NaP and 10,000 × *g* supernatant fluid and prepared with or without prior sonic treatment for 10 min, was analyzed on CsCl.

[g] Pooled 10,000 × *g* pellets from extraction 1 were suspended at 20% v/v in NaP, frozen, and re-extracted as extraction 1, except that sonic treatment was for 5 min.

or ribonuclease markedly reduced both thymidine and uridine radioactivity in CsCl (except in the region of intact virions for thymidine-labeled, infected cells). On the other hand, the CsCl patterns obtained with extracts from uninfected cells that had been labeled with ^{3}H-lysine did not change with nuclease treatment. Consequently, in further studies, no nuclease treatments were performed.

To evaluate further the contribution of nonvirion proteins to the amino acid radioactivity observed in CsCl cushions of SV40 from infected cells, two temperature-sensitive mutants isolated by P. Tegtmeyer were analyzed on CsCl cushions. Two classes of mutants have been reported not to synthesize viral particles at the restrictive temperature of 41 C (2). Mutant NTG-2 synthesized T antigen and viral DNA, whereas NTG-7 synthesized T antigen and induced host, but not viral, DNA replication. As shown in Fig. 2, infection with these mutants at 41 C resulted in CsCl cushion patterns similar to those obtained with uninfected cells.

Gel electrophoresis of viral particles isolated on CsCl cushion. A series of experiments was performed to ascertain the polypeptide components of the viral particles isolated on a CsCl cushion, to compare their composition to that obtained from purified viral particles, and to characterize comparable fractions of the CsCl cushion from uninfected cells.

Intact virions, labeled with ^{3}H-arginine and ^{14}C-valine, were purified from the medium of infected cells, as described above, and analyzed on SDS-acrylamide gels, as shown in Fig. 3. Four major proteins of 45,000 (I), 23,000 (II), 15,000 (IIIA), and 13,000 (IIIB) daltons were observed (immunoglobulin G H-chains, immunoglobulin G L-chains, and cytochrome *c* were used as marker standards; reference 19). A minor component was also seen at 35,000 on stained gels but was not resolved upon slicing the gel. This pattern was very similar to that reported recently by other workers (3, 5, 8). The possibility of additional minor components was not evaluated. A high-molecular-weight peak (80,000) varied in quantity and most likely represented an aggregate, as suggested by Estes et al. (5).

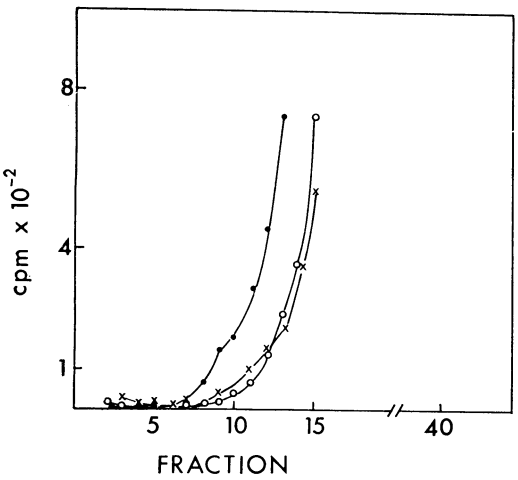

FIG. 2. *CsCl cushion analysis of extracts prepared from cells infected with temperature-sensitive mutants. Infected and uninfected cells were labeled with ^3H-lysine at 48 to 50 hr postinfection at 41 C. Samples were extracted and sedimented into CsCl cushions as in Fig. 1. Samples (25 μliters) of each CsCl fraction were assayed for radioactivity. The following preparations of 10,000 × g supernatant fluids were used: NTG-2 infected cells, 1.3 × 10^6 trichloroacetic acid-precipitable counts/min (●—●); NTG-7 infected cells, 1.5 × 10^6 counts/min (×—×); uninfected cells, 1.2 × 10^6 counts/min (○—○).*

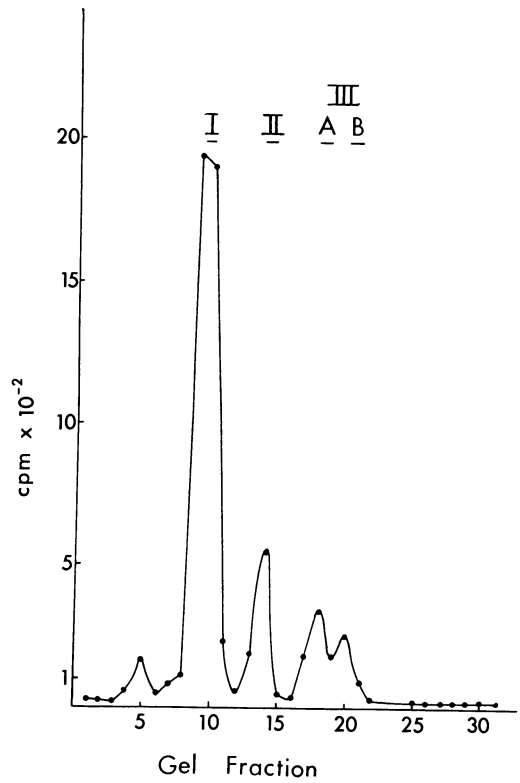

FIG. 3. *SDS-acrylamide gel analysis of purified intact virion. Radioactively labeled (^3H-arginine and ^{14}C-valine) virions were purified by repeated velocity and equilibrium centrifugation in CsCl and sucrose gradients as detailed in the text. Electrophoresis was performed on SDS-acrylamide gels, and radioactivity in 0.13-mm fractions was determined in NCS-Liquifluor as combined ^3H and ^{14}C.*

Table 3 shows the composite results (including arginine-valine ratios) of the gel patterns of polypeptides derived from intact virions and from empty shells (purified by the same procedure), and of the subviral components obtained from intact virions by alkaline disruption and sucrose gradient centrifugation by using the method of Anderer et al. (2). These results were in agreement with those reported by Estes et al. (5). Two points are worth emphasizing: (i) empty shells have a reduced proportion of the two lower-molecular-weight components (peaks IIIA and IIIB) and (ii) polypeptides IIIA and IIIB have a high arginine-valine ratio and are associated with DNA upon extraction, similar to the C protein reported by Anderer et al. (2).

The polypeptide composition of preparations from CsCl cushions obtained from infected and uninfected cells labeled with radioactive lysine are shown in Table 4. Intact virions had a polypeptide pattern consistent with purified, intact virions. There was no change in the distribution of radioactivity in the gel peaks with virions obtained on CsCl cushion alone or with subsequent equilibrium centrifugation (compare Table 4, lines 1, 2, and 4 with line 5). Empty shells isolated on CsCl cushions were contaminated with cellular proteins. Nonetheless, the predominant radioactive peak observed in gels was the major capsid protein (I). Cellular contamination appeared to be responsible for the relative increase in polypeptides III in the empty shell region. This interpretation was based on two observations: (i) 20 to 30% of the radioactivity in the "empty shell region" from uninfected cells migrated to the position of polypeptides III on gels (Table 4, line 3) and (ii) subsequent equilibrium centrifugation of empty shells resulted in a marked decrease in these polypeptides (Table 4, lines 4 and 5, and Fig. 4).

In summary, intact virions were clearly resolved on a CsCl cushion from empty shells and from cellular components. The radioactivity in the amino acid-labeled, intact virion peak varied with the experimental procedure but was routinely 1 to 5% of the total trichloroacetic acid-precipitable radioactivity in the sample analyzed on CsCl. Similar fractions from uninfected cells or from temperature-sensitive, mutant-infected cells were

TABLE 3. *Distribution of arginine and valine in purified viral components*

Prepn[a]	% Of total in viral proteins[b]							
	I	A/V[c]	II	A/V	IIIA	A/V	IIIB	A/V
Intact virions	65	3.7	12	8.8	10	10.8	6	6.8
Empty shells	75	3.0	8	3.0	3[d]		2[d]	
Capsid	61	3.3	7	3.2	6	3.7	7	5.0
DNA-complex	Nil		Nil		65[e]	8.2		

[a] Viral particles and components were purified as described in the text. "Capsid" was obtained as the 4 to 5*S* fraction and "DNA-complex" as the 46*S* fraction on sucrose gradients from alkaline-disrupted, intact virions.

[b] Per cent of total radioactivity recovered from sodium dodecyl sulfate-acrylamide gel; peaks as defined in Fig. 3. Sample counted in Nuclear-Chicago solubilizer-Liquifluor as combined ^{14}C and ^{3}H counts.

[c] Ratio of ^{3}H-arginine to ^{14}C-valine; double-label corrections were used.

[d] Counts too low for double-label determination.

[e] Sum in IIIA and IIIB.

TABLE 4. *Analysis of lysine-labeled cells*

Prepn[a]	CsCl Cushion		SDS-acrylamide gel[c] (% of total counts/min recovered in each polypeptide)							
	Intact virions (counts/min)	Empty shells (counts/min)	I		II		IIIA		IIIB	
			Intact virions	Empty shells	Intact virions	Empty shells	Intact virions	Empty shells	Intact virions	Empty shells
Experiment 1										
Infected, ^{3}H-lysine	4.8×10^5 (3.6)[b]	3.4×10^5 (2.6)[b]	74	60	6	4	6	3	4	6
Infected, ^{14}C-lysine	1.9×10^5 (3.5)	0.9×10^5 (1.7)	72	69	5	3	5	2	5	3
Uninfected, ^{3}H-lysine	0.1×10^5 (0.1)	1.2×10^5 (0.8)	14	7	13	11	6	8	6	23
Experiment 2										
Infected, ^{3}H-lysine	6.7×10^5 (2.5)	8.1×10^5 (3)	75	48	7	6	13[e]	28[e]		
			75[d]	79[d]	6	4	13[e]	6[e]		
Infected, ^{14}C-lysine	0.8×10^5 (2.3)	0.3×10^5 (1)	73		6		14[e]			

[a] Infected or uninfected cells, labeled with ^{14}C- or ^{3}H-lysine for 42 to 61 hr postinfection (MOI 25) in experiment 1 or for 41 to 65 hr postinfection (MOI 2) in experiment 2, were extracted by sonic treatment, and the 10,000 × *g* supernatant fluids were analyzed on CsCl cushion as described in the text.

[b] Values in parentheses represent per cent input, which equals (counts per minute in CsCl peak/trichloroacetic acid-precipitable counts per minute applied to cushion) × (100).

[c] Gel electrophoresis and recovery calculated as in Table 1..

[d] Samples purified by CsCl cushion and equilibrium centrifugation before gel analysis.

[e] Sum in peaks IIIA and IIIB.

0.2% or less. The degree of contamination of cellular radioactivity in the region of empty shells was considerably higher. However, only 7% of the nonviral contaminants of radioactive lysine was in the position of the major capsid protein of 45,000 daltons. Consequently, the radioactivity in the empty shell region could be assessed accurately by determining the percentage of total radioactivity in the major capsid protein on SDS-acrylamide gels.

Rate of synthesis of viral particles. The experiments described above demonstrate that the amount of viral particles synthesized in the presence of radiolabeled amino acid can be quantitated on CsCl cushions. The rate of synthesis of intact virions and of empty shells was then investigated.

Cells infected at an MOI of one were pulsed (72 hr postinfection) for 15 min to 2 hr with ^{3}H-lysine, and viral particles were analyzed on CsCl cushions (Fig. 5). At all time points, there was significantly more radioactivity in the region of empty shells; but the ratio decreased with time. The rate of incorporation of lysine into

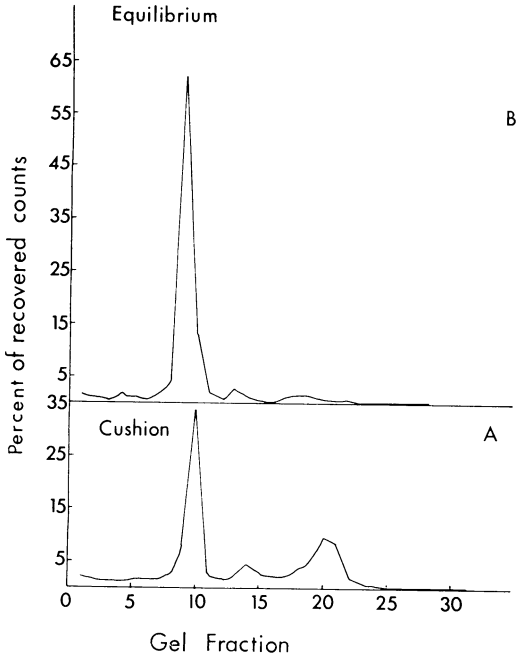

FIG. 4. *SDS-acrylamide analysis of empty shells. Electrophoresis was as described in the text. Samples were labeled with ^{3}H-lysine as in Table 4, experiment 2. A, empty shells prepared on CsCl cushion; B, empty shells prepared on CsCl cushion plus CsCl equilibrium centrifugation.*

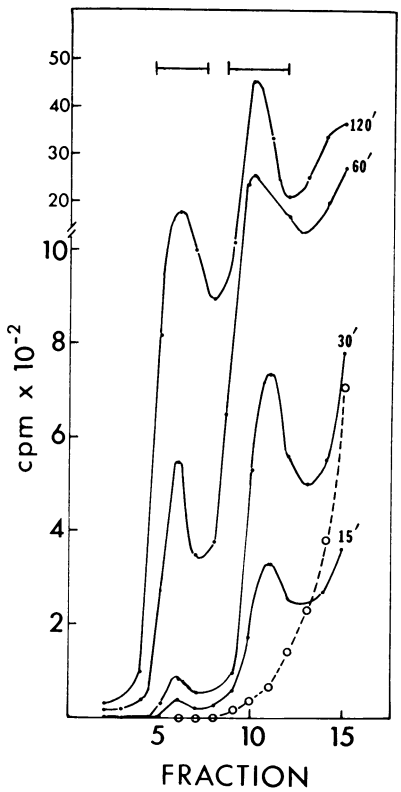

FIG. 5. *CsCl cushion analysis of pulse-labeled infected cells. Cells infected at an MOI of 1 were labeled with ^{3}H-lysine at 72 hr postinfection and analyzed on CsCl cushion. Samples (25 μliters) of the CsCl fractions were used to determine radioactivity. Uninfected cells (○--○), 120-min pulse, 56% of cell trichloroacetic acid-precipitable counts/min extracted into 10,000 × g supernatant fluid; supernatant fluid containing 1.2 × 10^6 trichloroacetic acid-precipitable counts/min analyzed on CsCl cushion. Infected cells (●—●), 15-min pulse, 55% counts/min recovered in 10,000 × g supernatant fluid, 1.4 × 10^5 counts/min analyzed; 30-min pulse, 85% counts/min recovered in 10,000 × g supernatant fluid, 2.4 × 10^5 counts/min analyzed; 60-min pulse, 77% counts/min recovered in 10,000 × g supernatant fluid, 6.5 × 10^5 counts/min analyzed; 120-min pulse, 64% counts/min recovered in 10,000 × g supernatant fluid, 1.3 × 10^6 counts/min analyzed.*

total cell trichloroacetic acid-precipitable material was linear from 15 min to 2 hr. In Fig. 6, the appearance of radioactivity in the two regions is plotted (after normalization of the data per milligram of protein in the 10,000 × *g* supernatant fluid analyzed.) In view of the probability that empty shells were more likely to be contaminated with cell material, all samples were analyzed on SDS-acrylamide gels to determine the proportion of radioactivity actually associated with the major capsid protein (I). In all cases, this value was greater than 60%, ranging from 65 to 75% (compared to the 70 to 80% expected for particles purified by equilibrium centrifugation). Only 15% of the radioactivity in the empty shell region of the CsCl cushion prepared from uninfected cells pulsed for 2 hr had the same mobility on gels as did "capsid protein I." Analysis of the medium after concentration with polyethylene glycol (6) ruled out the possibility that large amounts of intact virions were preferentially lost as extracellular virus during the pulse.

Predominant labeling of empty shells in short pulse (1 to 3 hr) was observed in several experiments under a variety of conditions, which involved 70-fold differences in multiplicity of infection, different virus pools, inclusion of temperature-sensitive mutants at the permissive temperature (32 C), and different times postinfection (24 to 96 hr). In contrast, prolonged labeling periods (16 to 24 hr) routinely resulted in more radioactivity in intact virions (Tables 4 and 5). Since it had been found previously that empty shells were more prominent in preparations submitted to heavy sonic treatment (Table 1), the percentage of V antigen, trichloroacetic acid-

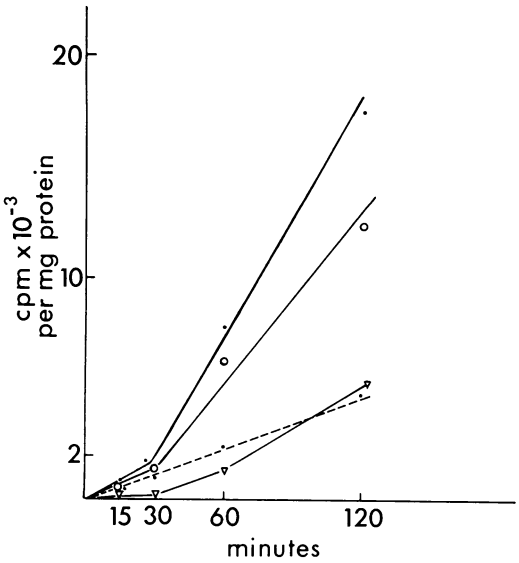

FIG. 6. *Particle synthesis. Peaks pooled as indicated by bars at top of Fig. 5 and normalized per milligram of cell extract protein analyzed on CsCl cushion: total particles* (●—●), *empty shells* (○—○), *intact virions* (△—△), *total cellular trichloroacetic acid-precipitable counts/min* × 10^{-3} (●--●).

precipitable radioactivity, and protein released by sonic treatment is included. The degree of variability in the yield of the two types of viral particles among experiments was appreciable; however, it did not appear to correlate with any of the parameters mentioned. Within experiments, there was good agreement in the proportion of the two particles in nearly every case.

An estimate of the rate of accumulation of viral particles relative to the synthesis of total cell protein can be made from the data for 2-hour pulses shown in Table 5. For example, it can be seen that the ^{3}H-lysine incorporated into viral particles increases with multiplicity and time postinfection, reaching as much as 5 to 10% of the total trichloroacetic acid-precipitable radioactivity extracted with sonic treatment.

Effect of AraC on virus assembly. In an attempt to dissociate synthesis of the two particles, the effect of AraC late in infection was studied. At 42 and 48 hr postinfection, the medium was removed and replaced with lysine-free medium containing 10^{-4} M AraC (a concentration sufficient to inhibit DNA synthesis in infected cells by 95% within 1 hr). Four hours later, ^{3}H-lysine and ^{14}C-thymidine were added for 2 hr. Parallel, control-infected cultures were pulse-labeled either at the time of addition of AraC (experiment 1) or after 4 hr of incubation in the lysine-free medium without AraC (experiment 2). In all cases, intact virions and empty shells were isolated on CsCl cushions, and the proportion of radioactivity in the major capsid protein was determined for each sample. All data were normalized on the basis of total trichloroacetic acid-precipitable radioactivity in the sample to adjust for possible changes in rates of incorporation of ^{3}H-lysine into cell protein. As shown in Table 6, there was a 50% inhibition in the rate of synthesis or rate of assembly of intact virions, or both, with little change in the rate of appearance of empty shells.

DISCUSSION

A procedure which permits rapid analysis of virus assembly in SV40-infected cells labeled with radioactive amino acids has been described. Under the conditions of extraction used (sonic treatment with subsequent treatment by NP40), discrete peaks corresponding to intact virions and empty shells were observed in a CsCl cushion of 1.32 g/cm^3. Comparison of extracts of wild-type infected cells, uninfected cells, and cells infected with temperature-sensitive mutants on CsCl cushions with subsequent SDS-acrylamide gel electrophoresis was performed to determine the degree of contamination of the viral particles. Intact virions were contaminated only minimally with ^{3}H-lysine cellular proteins. The region of empty shells contained various amounts of cellular proteins, usually ranging from 15 to 30% of the total radioactivity in the fraction. On SDS-acrylamide analysis, only a minor proportion (7 to 15%) of the ^{3}H-lysine in that region from uninfected cells migrated in the area of the major capsid protein (45,000 daltons). Thus, accurate determination of the synthesis of empty shells could be obtained by combining CsCl cushion and gel electrophoresis. By using a 2-hr pulse with ^{3}H-lysine, the rate of synthesis of viral particles was determined at different MOI values and times postinfection (Table 5). Late in infection and at high multiplicities, 5 to 10% of the total cellular acid-precipitable ^{3}H-lysine was isolated in viral particles.

The relationship between the two major classes of viral particles was investigated by analysis on the CsCl cushions. The intensity of sonic treatment influenced markedly the proportion of the two particles when the infected cells were radioactively labeled for long periods (48 to 61 hr postinfection). Under these conditions, the amount of empty shells extracted increased with the duration of sonic treatment. This result did not appear to be attributable to breakdown of intact virions during the extraction, since reconstruction experiments with admixed intact virions failed to demonstrate appreciable conversion of intact virions to empty shells. Though it is not

TABLE 5. *Proportion of lysine incorporation into viral particles*[a]

Virus pool	MOI[b]	Labeling period (hr postinfection)[c]	% Of total recovered in 10,000 × g supernatant fluid			CsCl cushion (% input)[e]	
			CFU[d]	Counts/min	Protein	Intact virion	Empty shells
Short label							
13	1	48–50	33	42	42	0.2	0.3
		68–70	40	50	50	0.6	1.3
14	70	24–26	56	56	49	0.4	1.0
		48–50	52	43	47	2.5	3.7
		68–70	58	34	47	3.5	4.4
14	7	48–50	86	57	60	1.7	3.5
		68–70	85	59	57	2.3	3.9
13 + 14	35	48–50	86	63	64	3.3	4.5
		68–70	85	54	58	3.6	8.5
Long label							
13	1	49–62	80	60	ND[f]	0.5	0.6
		71–79	80	78	ND	3.8	1.1
		79–95	75	58	ND	2.2	1.0

[a] Samples prepared as in Table 4.
[b] Multiplicity of infection.
[c] ^{3}H-lysine used in all experiments except as noted.
[d] Complement-fixation units.
[e] Calculated as in Table 4.
[f] Not determined.

TABLE 6. *Effect of cytosine-β-D-arabinofuranoside (AraC) on virus assembly*[a]

Expt	Hr postinfection		% Inhibition[b] of		Intact virion-empty shell ratio[c]	
	AraC added	^{3}H-lysine added	Intact virion	Empty shells	Control	AraC
1[d]	42–48	46–48	48	12	0.66	0.28
2[e]	48–54	52–54	49	14	0.51	0.30

[a] Cells infected at MOI of 50 were labeled for 2 hr in lysine-free medium with ^{3}H-lysine (10 μCi/ml) and ^{14}C-thymidine (0.5 μCi/ml). Intact virions and empty shells were isolated on CsCl cushion as in Fig. 1. The pooled peaks were analyzed on sodium dodecyl sulfate-acrylamide gels for the proportion of major capsid protein.
[b] Calculated as follows: 100 − (% trichloroacetic acid-precipitable radioactivity in major capsid protein of viral particles in AraC-treated cells × 100) divided by (% trichloroacetic acid-precipitable radioactivity in the major capsid protein in viral particles in control-infected cells).
[c] Per cent trichloroacetic acid-precipitable radioactivity in major capsid protein of intact virions divided by % trichloroacetic acid-precipitable radioactivity in major capsid protein of empty shells.
[d] Control labeled at 42 to 44 hr postinfection.
[e] Control incubated in lysine-free medium from 48 to 54 hr postinfection and labeled at 52 to 54 hr postinfection.

possible at present to define precisely the origin of empty shells, they are likely heterogeneous in biological origin as well as structure (as determined by electron microscopy). Two possibilities were explored in this investigation: (i) that empty shells were synthesized independently of intact virions and (ii) that empty shells resulted from breakdown of previously synthesized, intact virions. A third possibility—that empty shells were precursors of intact virions—is discussed in the accompanying paper.

Two lines of evidence support the first possibility. First, in experiments in which relatively short pulses were used to label particles, ^{3}H-lysine was preferentially incorporated into empty shells. This was observed over a wide range of experimental conditions, including different intensities of sonic treatment (Table 5). In two experiments (one not shown), the proportion of radioactivity recovered in intact virions increased with longer pulses between 15 min and 2 hr. If breakdown were responsible, the lower radioactivity in intact virions would require extraordinary instability of recently synthesized virus, which would decrease concurrently with the duration of pulse over a relatively short period of time. The second line of evidence involves experiments in which the DNA inhibitor AraC was observed to dissociate synthesis of the two viral particles. Late in infection, after the onset of appearance of virus particles, there was significant pooling of unassembled, viral DNA (H. L. Ozer, *unpublished data*). Interruption of new DNA synthesis still permitted the synthesis of viral particles. However, the rate of synthesis of viral particles began to decrease concurrently with duration of incubation in AraC. After 4 hr in AraC, the rate of synthesis of intact virions had fallen to 50% of the control level. The synthesis of empty shells, on the other hand, had decreased only 10%. These results suggest that empty shells were less dependent on viral DNA for formation than were intact virions, which is

consistent with either the first or third possibility described above.

Studies on the polypeptide composition of empty shells suggest that they may also result from breakdown of intact virions. Empty shells obtained by repeated velocity and equilibrium centrifugation in CsCl in this investigation and in that of Estes et al. (5) have been found to contain reduced but significant quantities of low-molecular-weight components (polypeptides III) when compared to that found in intact virions. These polypeptides have a high arginine-valine ratio and can be extracted from virions in association with viral DNA. Consequently, the presence of these proteins may reflect the prior presence of DNA in empty shells. The recent report by Koch et al. (10), indicating that the C protein (DNA-complex protein) can be detected on the surface of intact virions and empty shells, is consistent with this interpretation, since they did not evaluate the deoxyribonuclease susceptibility of the purified, intact virions. Empty shells isolated on CsCl cushions in this study were found to contain appreciable quantities of polypeptides III. However, the majority of this material appeared to be attributable to cellular contamination. Of the radioactivity in the region of empty shells prepared from uninfected cells, 20 to 30% migrated in the region of polypeptides III on SDS-acrylamide gels. Furthermore, subsequent equilibrium centrifugation in CsCl of the empty shells resulted in a marked decrease in the proportion of these polypeptides.

In conclusion, it is emphasized that the assay of viral particles on a CsCl cushion of 1.32 to 1.33 g/cm^3 should be applicable to a variety of experimental manipulations to evaluate radioactive virion formation, including situations in which the presence of previously synthesized particles precludes the use of available immunological and virological techniques. This procedure has been employed to identify different classes of noncomplementing, temperature-sensitive mutants of SV40 by the nature of particles synthesized (23). In the accompanying report, this procedure, together with electrophoresis on SDS acrylamide gels, was used to determine the rate of synthesis of the major capsid protein (I).

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