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Effect of Phleomycin on Polyoma Virus Synthesis in Mouse Embryo Cells

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The addition of phleomycin (25 μg) to primary mouse embryo cells infected with polyoma virus was found to cause 96% inhibition of the synthesis of infectious virus. When ribonucleic acid and protein synthesis was investigated in these cells by use of isotope incorporation, it was found that neither was inhibited drastically. Immunofluorescent staining studies with the use of antibody directed to the viral structural proteins showed that proteins were synthesized in the presence of the antibiotic. However, when deoxyribonucleic acid (DNA) synthesis was investigated, it was found that DNA synthesis in uninfected cells was completely inhibited within the initial 10 hr of phleomycin addition, whereas DNA synthesis in infected cells proceeded at a reduced rate. Selective DNA extraction (Hirt method) of phleomycin-treated infected cells demonstrated that synthesized viral DNA was salt-extractable, similar to that in infected control cells lacking phleomycin. This extracted DNA was further fractionated by ethidium bromide-cesium chloride density gradient equilibrium centrifugation. The phleomycin-treated preparations revealed twice as much component II (circular nicked and linear) as component I (supercoiled) DNA, whereas the DNA from normally infected control cells showed the reverse picture. It was also demonstrated that viral particles synthesized in the presence of phleomycin did not contain component I DNA. This packaged DNA was found to consist of fragments of both the host and viral types. Cells that were prelabeled with ^{3}H-thymidine and then treated with phleomycin demonstrated host DNA degradation. However, fragments formed from prelabeled host DNA were not encapsidated into viral particles.

Phleomycin, a copper-containing water-soluble antibiotic, was isolated from a culture filtrate of *Streptomyces verticillus* (13) and was later found to have antitumor activity (2, 10, 19). In cultures of mammalian cells and bacteria, it selectively inhibited deoxyribonucleic acid (DNA) synthesis (19). Falaschi and Kornberg (7) demonstrated that phleomycin is preferentially bound to adenine-thymine base pairs. Phleomycin was also effective in blocking the synthesis of infectious φX 174 in *Escherichia coli* (17) and simian virus 40 in primary green monkey kidney cells, but did not affect the replication of ribonucleic acid (RNA)-containing poliovirus in the same cells (20). Recently Grigg (8) reported that phleomycin induced breakdown of DNA and subsequent death in *E. coli* and that excision-less mutants were insensitive to phleomycin.

The present report deals with the effects of phleomycin on polyoma virus synthesis in mouse embryo cells, and primarily with its effects on viral DNA synthesis.

MATERIALS AND METHODS

Tissue culture. Throughout the investigation, primary cultures of mouse embryo cells prepared as described previously (11) were used. The cells were grown to confluent monolayers in Eagle's (6) minimal essential medium (MEM) supplemented with 5% fetal calf serum (FCS). Infected cultures were maintained in Eagle's MEM supplemented with 5% dialyzed FCS.

Virus. The wild-type strain of polyoma virus originally obtained from S. Stewart, National Institutes of Health, was used in these studies. Virus stocks used in these experiments contained 5×10^8 to 9×10^8 plaque-forming units (PFU)/ml.

Phleomycin. Phleomycin (lot A9331-648) was supplied by Bristol Laboratories, Syracuse, N.Y. It was dissolved in Eagle's medium at a concentration of 1 mg/ml, filter-sterilized (0.45 μm membrane filter; Millipore Corp., Bedford, Mass.), distributed into small screw-capped tubes, and stored frozen at −20 C.

Virus purification and quantitation. Polyoma virus was purified as described previously (3). Virus particle

concentration was established by the hemagglutination (HA) test, and the infectivity of these preparations was determined by plaque assay (PFU). The techniques employed for these assays have been reported (3).

Radioisotopic incorporation of precursors. Uninfected and infected cultures were incubated (33 hr postinfection) in Eagle's medium containing 1 μCi of ^{3}H-thymidine (DNA), ^{3}H-uridine (RNA), or ^{3}H-valine (protein) per ml, with or without phleomycin (25 μg/ml). To suppress the possible interconversion of labeled nucleosides, 5 μg of uridine/ml was added to the Eagle's medium containing ^{3}H-thymidine, and the same amount of thymidine was added to the medium containing ^{3}H-uridine. Cultures were harvested at various times, the radioactive medium was removed, the cells were washed once with cold phosphate-buffered saline (PBS), and then cold trichloroacetic acid (5% final concentration) was added to each culture. The cells were then stored at 4 C overnight after which they were washed three times with cold 5% trichloroacetic acid to remove unincorporated radioactivity. Cells were dissolved in 1 N NaOH, and radioactivity (counts per minute) and protein were determined.

Radioisotopic incorporation (pulse). Uninfected and infected cultures that were maintained in Eagle's medium with or without phleomycin (25 μg/ml) were pulsed for 1 hr with ^{3}H-thymidine (4 μCi/ml) 2 hr prior to harvesting. The radioactive medium was removed and the cells were washed twice with PBS. The cultures were further incubated for 1 hr in unlabeled Eagle's medium before they were harvested, to deplete the ^{3}H-thymidine phosphate cell pool.

Selective extraction of polyoma DNA. Pulse-labeled cultures were washed once with PBS, and the cells were lysed by adding sodium dodecyl sulfate (SDS); viral DNA was selectively extracted with 1.0 M NaCl (9). The supernatant containing the viral DNA was dialyzed overnight at 4 C against tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer [0.01 M Tris, *p*H 7.5; 0.01 M ethylenediaminetetraacetate (EDTA)] and then was further fractionated by gradient centrifugation or radioactive incorporation (counts per minute) was directly determined.

Separation of DNA by isopycnic centrifugation in ethidium bromide-cesium chloride. DNA extracted from cells by the Hirt method (9) or polyoma DNA obtained from purified complete virus particles (14) was separated into component I (supercoiled) and a component II mixture (nicked circular plus linear DNA) on ethidium bromide-cesium chloride density gradients by centrifuging to equilibrium (18).

Fluorescence microscopy. The synthesis of viral structural protein was assayed by the indirect immunofluorescence technique. Infected cover-slip cultures were harvested 30 hr after infection and stained as previously described (11). The preparations were examined for nuclear fluorescence with the Lietz ortholux fluorescence microscope.

Incorporation of ^{3}H-valine into polyoma virus. Polyoma virus was labeled by maintaining infected cultures in Eagle's medium containing ^{3}H-valine (3 μCi/ml) from 5 hr until 40 hr postinfection. Phleomycin (25 μg/ml) was added sequentially at 5, 10, 15, and 20 hr after infection to respective cultures. All cultures were harvested at 40 hr postinfection, and the virus was purified. Fractions were collected from cesium chloride density gradients and assayed for radioactivity and HA titer.

Preparation of ^{3}H-thymidine-labeled DNA from purified virus. Infected cultures were incubated in Eagle's medium containing ^{3}H-thymidine (3 μCi/ml) with or without phleomycin (25 μg/ml) from 5 hr until 40 hr postinfection. Three times as many cultures were prepared for the phleomycin-treated preparation to obtain enough virus particles for the subsequent analysis of the encapsidated DNA. Virus was harvested at 40 hr postinfection and was purified. Fractions from cesium chloride density gradients which contained ^{3}H-thymidine-labeled virus were pooled for both control and phleomycin-treated preparations, and the viral samples were dialyzed against Tris-hydrochloride buffer (0.01 M, *p*H 7.5) to remove cesium chloride. The viral particles were then subjected to alkaline degradation of the polyoma capsids to release DNA (14).

DNA-DNA hybridization. Component I and II polyoma DNA was obtained from ethidium bromide-cesium chloride gradients as described above. DNA from purified polyoma particles was obtained as described above. The mouse embryo cell DNA fraction was extracted with redistilled phenol saturated with 1 M NaCl, Tris-hydrochloride buffer (0.01 M, *p*H 7.2), and 0.01 M EDTA. Phenol was removed by dialysis against Tris-hydrochloride (0.01 M, *p*H 7.2)-EDTA (0.01 M) buffer. DNA-DNA hybridization was performed by the procedure of Cuzin et al (4).

Alkaline sucrose density gradient velocity centrifugation. Sucrose density gradient centrifugation was performed by sedimenting 0.1 ml of the sample through an alkaline linear sucrose gradient (5 to 20% sucrose, 0.1 M NaOH, 0.9 M NaCl; bottom of the tube contained a cushion of 0.3 ml of 1.70 g/ml cesium chloride in 20% sucrose) in an SW 50.1 rotor for 4.5 hr at 130,000 $\times$ *g*. Samples were collected through a hole punctured in the bottom of the tube onto Whatman 3MM filter pads (2.3 cm). The filter pads were dried and were washed twice with cold 10% trichloroacetic acid and once with cold acetone. The above dried filters were placed in scintillation vials, a toluene spectrafluor mixture was added, and radioactivity was determined in a scintillation counter.

Prelabeling of host DNA. Mouse embryo cells were seeded and grown in Eagle's medium containing either ^{3}H-thymidine (1 μCi/ml) or ^{14}C-thymidine (0.25 μCi/ml). The cells were grown to monolayers (three generations) in the radioactive medium; 2 hr before infection, the radioactive medium was removed, and the cells were washed three times with PBS and incubated further in unlabeled medium until infection. By means of autoradiography, it was determined that 90 to 95% of the cells were labeled in the nucleus. To determine prelabeled host DNA degradation, infected cultures were maintained in Eagle's medium with or without phleomycin (25 μg/ml). The medium was collected at various intervals and the cells were dissolved in 1.0 N NaOH. Radio-

TABLE 1. *Effect of phleomycin on synthesis of infectious polyoma virus*

Conditions[a]	PFU/ml	Synthesis
		%
No phleomycin, harvested at 15 hr	6.2×10^6	0
No phleomycin, harvested at 48 hr	2.5×10^8	100
Phleomycin (25 μg/ml) added at		
4 hr	1.7×10^7	4
10 hr	2.2×10^7	7
15 hr	2.3×10^7	7
20 hr	7.2×10^7	27
30 hr	1.0×10^8	39

[a] All cultures to which phleomycin was added were harvested at 48 hr postinfection.

activity was determined for medium and cell hydrolysate, and the percentage (of total) of radioactivity appearing in the medium was calculated.

Other quantitative techniques. Protein was assayed according to the method of Lowry et al. (12). Radioactivity was measured in a Packard liquid scintillation counter.

Radioisotopes. Protein was labeled with ^{3}H-L-valine (specific activity, 0.50 Ci/mmole), RNA was labeled with uridine-*6-^{3}H* (specific activity, 10 to 20 Ci/mmole), and DNA was labeled with methyl-^{3}H-thymidine (specific activity, 10 Ci/mmole). The radioisotopically labeled compounds were purchased from Schwarz BioResearch, Inc., Orangeburg, N.Y.

RESULTS

Effect of phleomycin on synthesis of infectious polyoma virus. The quantity of polyoma virus synthesized in mouse embryo cells was compared with that made in infected cultures which received phleomycin (25 μg) at various times during a 48-hr infection period. As shown in Table 1, phleomycin inhibited the synthesis of infectious virus 96% when it was added to the culture during the initial 4 hr of infection, and was quite effective if added prior to 15 hr of infection (over 90% inhibition). The degree of inhibition decreased thereafter, although its effect was obvious as late as 30 hr postinfection (60% inhibition).

Effect of phleomycin on the incorporation of radioisotopic precursors into DNA, RNA, and protein of mouse embryo cells. This experiment clearly indicates that phleomycin was more effective in inhibiting DNA synthesis in normal mouse embryo cells (Fig. 1A) than in infected cultures (Fig. 1B). After 10 hr, there was no further incorporation of isotope in uninfected cells in the presence of the drug (Fig. 1B). The antibiotic did not significantly affect either RNA (Fig. 1C and D) or protein synthesis (Fig. 1E and F) in either uninfected or polyoma-infected cultures.

Selective salt extraction of polyoma DNA. Since host DNA synthesis was more effectively inhibited by phleomycin in uninfected cells than in infected cells (Fig. 1A and B), it was of interest to investigate further the kind of DNA being synthesized in phleomycin-treated infected cells.

Uninfected and polyoma-infected mouse embryo cells were pulse-labeled with ^{3}H-thymidine at various times during a 30-hr infection period and compared with similar cultures that were treated with the antibiotic. The viral DNA in these cultures was then selectively extracted by the Hirt (9) method. It was found that virus-infected cells treated with phleomycin were still capable of synthesizing DNA soluble in 1 M NaCl (Fig. 2B). The rate of DNA synthesis was maximal 22 to 23 hr after infection. At this interval, treated cultures allowed 60% (25 μg/ml) and 50% (50 μg/ml) DNA synthesis when compared with untreated polyoma-infected cells. There was very little Hirt-extractable DNA in either untreated or phleomycin-treated uninfected cultures.

The salt-soluble DNA (Fig. 2B) synthesized 22 to 23 hr postinfection was further fractionated by ethidium bromide-cesium chloride density gradient centrifugation (Fig. 3). It was found that addition of phleomycin to the culture caused a drastic decrease in component I DNA but did not affect the synthesis of the DNA appearing as component II. Consequently, the ratio of component II to component I for phleomycin-treated preparations was much greater than that for untreated samples: 0.69 for untreated (Fig. 3A), 1.91 for 25 μg of phleomycin (Fig. 3B), and 5.93 for 50 μg of phleomycin (Fig. 3C)-treated samples. The per cent inhibition of component I DNA synthesis was 65% (25 μg/ml) and 89% (50 μg/ml) for phleomycin-treated preparations (Fig. 3B and C). It was also noted that the molecular species of component II DNA in phleomycin-treated preparations appeared to be more homogeneous judging from the sharpness of the band.

Effect of phleomycin on synthesis of polyoma virus antigen. Since the ^{3}H-valine incorporation experiment (Fig. 1E and F) demonstrated that protein synthesis was virtually unaffected by the antibiotic, an experiment was performed to determine whether the antibiotic allowed synthesis of polyoma structural proteins (antigens). As shown in Table 2, approximately 50% virus antigen synthesis occurred when phleomycin was added between 1 and 15 hr after infection. The effect of phleomycin decreased rapidly when addition of phleomycin was delayed.

^{3}H-valine incorporation into purified polyoma

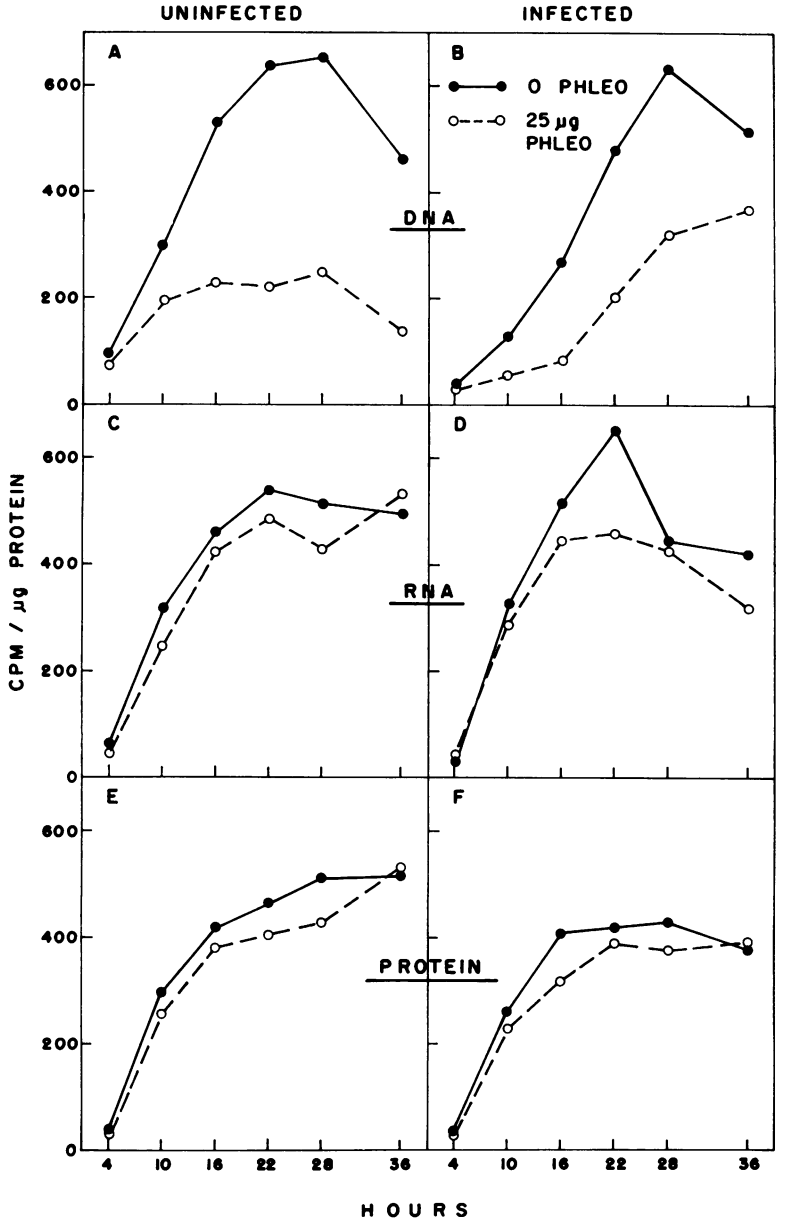

FIG. 1. *Effect of phleomycin on the incorporation of radioisotopic precursors into DNA, RNA, and protein. Uninfected and infected cultures were maintained in Eagle's medium containing 1 µCi of ^{3}H-thymidine (DNA), ^{3}H-uridine (RNA), or ^{3}H-valine (protein) per ml, with or without phleomycin (25 µg/ml). The medium containing the respective label and drug was added 3 hr postinfection. Cultures were harvested at the times indicated and specific activities (counts per minute per microgram of protein) were determined.*

virus after sequential addition of phleomycin. The findings that some synthesis of both polyoma DNA (component I) and virus antigen occurred in the presence of phleomycin, despite the fact that synthesis of infectious virus was inhibited (96%) under the same conditions, made it necessary to investigate whether viral particles were being formed. As shown in Fig. 4, both complete and incomplete virus particles were synthesized in the presence of phleomycin, and as addition of phleomycin was delayed the effect of phleomycin was drastically reduced. When phleomycin was

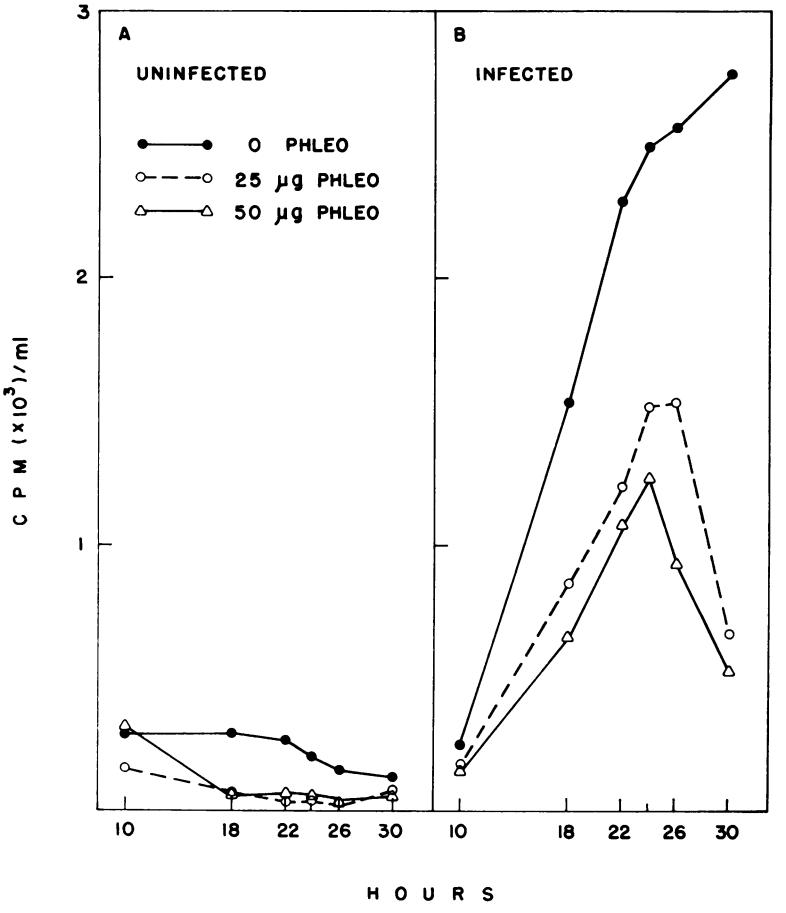

FIG. 2. *Selective salt extraction of DNA from untreated and phleomycin-treated cultures. Uninfected (A) and polyoma-infected (B) cultures were maintained in Eagle's medium with (25 or 50 μg/ml) or without phleomycin. Appropriate cultures were exposed to ^{3}H-thymidine (4 μCi/ml) for 1 hr at the indicated intervals. The radioactive medium was removed and the cells were washed. The cultures were further incubated for 1 hr in unlabeled Eagle's medium to deplete the ^{3}H-thymidine phosphate cell pool. These cultures were then harvested and viral DNA was extracted as described (9).*

added 20 hr postinfection, there was no apparent effect on total ^{3}H-valine incorporation into virus particles. By summing the radioactive counts under each peak, and determining the ratio of incomplete virus peak to complete virus peak, it was found that the ratio was the lowest (1.18) for control (Fig. 4A) and the highest (1.91) for the sample to which phleomycin was added at 5 hr postinfection (Fig. 4B). The ratios of the three other samples (Fig. 4C, D, and E) fell between two extremes according to the order of phleomycin addition. The synthesis of complete virus was found to be more sensitive to phleomycin inhibition, indicating the possibility that the availability of the viral DNA could be the limiting factor.

Characterization of the DNA in purified virus particles assembled in the presence of phleomycin. Since phleomycin is an inhibitor of DNA synthesis, the most likely candidate responsible for the defectiveness of complete virus would be the DNA. Therefore, an attempt was made to determine the nature of the DNA packaged in virus particles previously grown in the presence of phleomycin. Recently, Perry et al. (14) developed a simple and effective method to degrade polyoma virus and to fractionate component I and component II DNA from purified polyoma particles in ethidium bromide-cesium chloride gradients. This technique was used to compare the nature of the DNA packaged in polyoma particles that were previously grown in cultures that were untreated

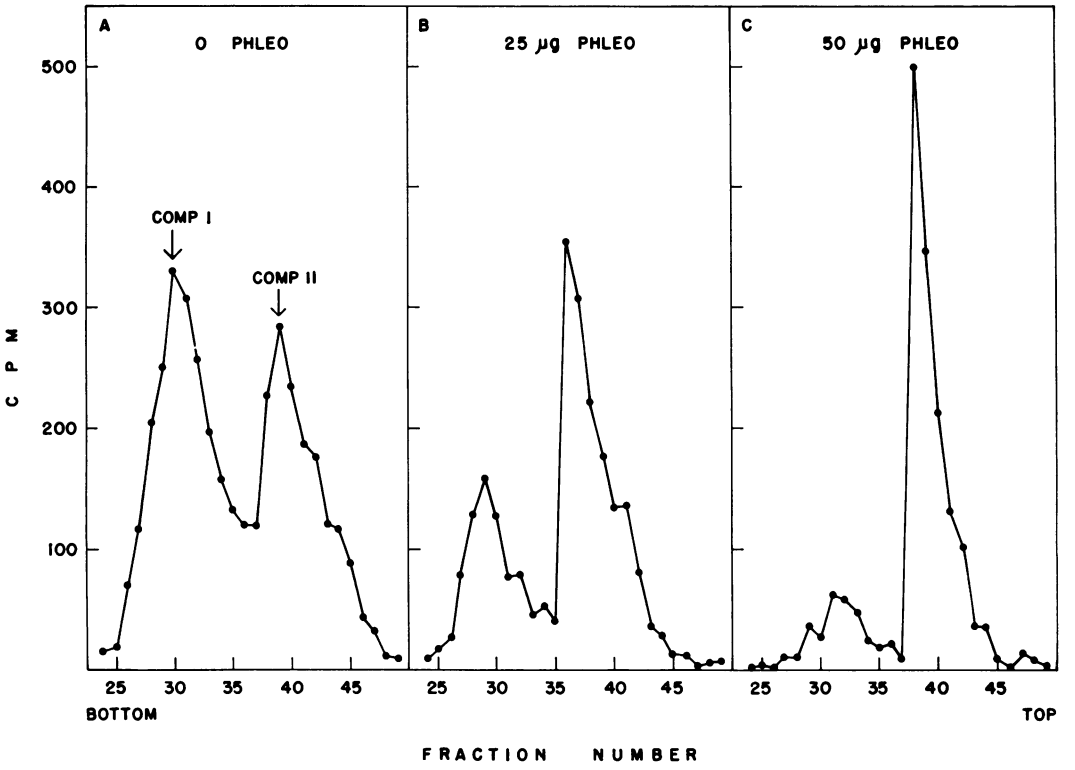

FIG. 3. *Nature of DNA extracted by the Hirt method. The salt-extracted DNA from infected cultures (Fig. 2B) pulse-labeled 22 to 23 hr postinfection was further fractionated by ethidium bromide-cesium chloride density gradient centrifugation. A sample (0.85 ml) of the DNA was mixed with 2 ml of cesium chloride (1.805 g/ml in Tris buffer, 0.02 M, pH 7.5) and ethidium bromide (final concentration, 100 μg/ml). The solution was mixed by gently inverting the tube, and the preparation was overlaid with mineral oil (2.1 ml) and centrifuged to equilibrium in an SW 50.1 rotor at 175,000 × g for 24 hr at 20 C. Four-drop fractions were collected directly into scintillation vials containing 10 ml of aqueous scintillation fluid or into tubes containing 0.1 ml of Tris buffer (0.01 M, pH 7.5; EDTA, 0.01 M), and radioactivity was determined in a scintillation counter. (A) DNA from untreated infected culture; (B) DNA from phleomycin (25 μg/ml)-treated infected culture; (C) DNA from phleomycin (50 μg/ml)-treated infected culture.*

TABLE 2. *Effect of phleomycin on the synthesis of polyoma virus antigens*

Conditions[a]	Nuclear fluorescence	Antigen synthesis
	%	%
Normal mouse cells	0.0	0.0
Infected cells, no phleomycin	52.0	100.0
Phleomycin (25 μg/ml) added at		
1.5 hr	25.4	48.9
5 hr	27.6	53.1
10 hr	27.4	52.7
15 hr	27.8	53.4
20 hr	35.5	68.3
25 hr	46.0	88.5

[a] All cultures were harvested at 30 hr postinfection. Average cell number counted: 150.

or treated with phleomycin. A striking difference between the two preparations was found; the DNA from polyoma virus (Fig. 5A) yielded more than 50% of the total counts per minute in the component I peak, whereas the DNA from purified particles previously grown in the presence of phleomycin contained only component II DNA (Fig. 5B).

Alkaline sucrose density gradient studies. The DNA from purified polyoma particles obtained from phleomycin-treated cultures was also investigated by sedimentation through alkaline sucrose. This DNA preparation was compared with polyoma DNA which was closed circular supercoiled (component I) and with circular nicked DNA (component II). As can be seen in Fig. 6, the component I marker showed a characteristic 53*S* value and component II and 18*S*

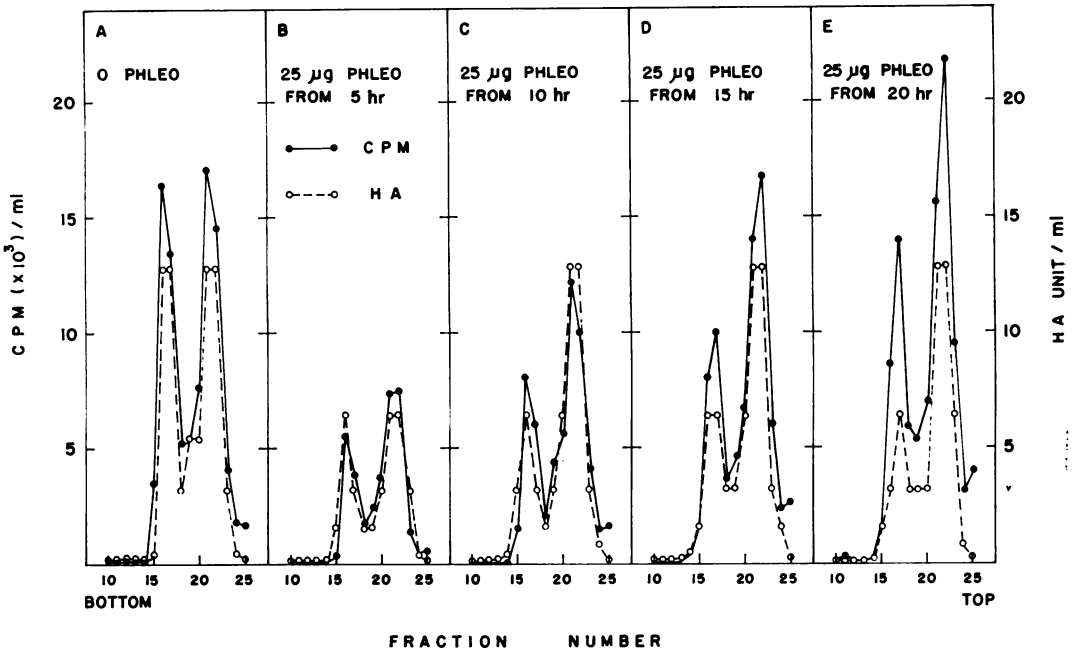

FIG. 4. *Effect of phleomycin on the synthesis of ^{3}H-valine-labeled polyoma virus. Polyoma virus was labeled by maintaining infected cultures in Eagle's medium containing ^{3}H-valine (3 µCi/ml) from 5 hr until 40 hr postinfection. Phleomycin (25 µg/ml) was added sequentially at 5 (B), 10 (C), 15 (D), and 20 hr (E) after infection; these cultures were compared with a group of cultures that did not receive phleomycin (A). All cultures were harvested at 40 hr postinfection, and virus was purified by CsCl density gradient centrifugation as described (3). Fractions were collected and assayed for hemagglutination activity and the incorporation of ^{3}H-valine into viral particles (counts per minute).*

value. However, the DNA from the purified polyoma particles obtained from phleomycin-treated cultures demonstrated a sedimentation pattern characteristic of fragmented DNA.

DNA-DNA hybridization. The alkaline sucrose gradient study of the DNA from purified particles obtained from phleomycin-treated cultures indicated DNA fragmentation or possibly the encapsidation of host DNA synthesized during infection. To determine the nature of the DNA in these viral particles, DNA-DNA hybridization was employed. As can be observed in Table 3, the cross-reactions of viral and cellular DNA with the DNA from the viral particles obtained from phleomycin-treated cultures were equal. It is concluded that these virions contain fragmented viral and host DNA (Fig. 6). This finding demonstrates the probable reason for the occurrence of noninfectious particles after phleomycin treatment.

Degradation of prelabeled host DNA by phleomycin. Since it was shown in the above experiments that phleomycin allowed packaging of host DNA synthesized during virus infection, it was also of interest to determine whether the drug caused fragmentation of host DNA synthesized prior to infection. To investigate the possibility of host DNA degradation by phleomycin, mouse embryo cultures prelabeled with ^{3}H-thymidine were maintained in an unlabeled medium with or without phleomycin. The appearance of ^{3}H-label in the medium was examined by collecting the medium at various times during a 36-hr infection period. As shown in Fig. 7, prelabel (^{3}H) counts appearing in the medium were increased markedly by phleomycin treatment, especially in infected cultures; there was a 56% increase in uninfected (Fig. 7A) and a 74% increase in infected (Fig. 7B) cultures at 36 hr postinfection.

The preceding experiment clearly showed that accelerated host DNA degradation was occurring in the presence of phleomycin. However, since our concern was to find a possible involvement of host DNA fragments in component II DNA, salt (Hirt method) extraction of double-labeled DNA (^{14}C-thymidine prelabel, ^{3}H-thymidine postlabel) from infected cultures and subsequent separation of the DNA into component I and component II DNA was performed. The ^{14}C-prelabeled infected cultures were pulse-labeled for 1 hr at various times during a 28-hr infection. The cultures were

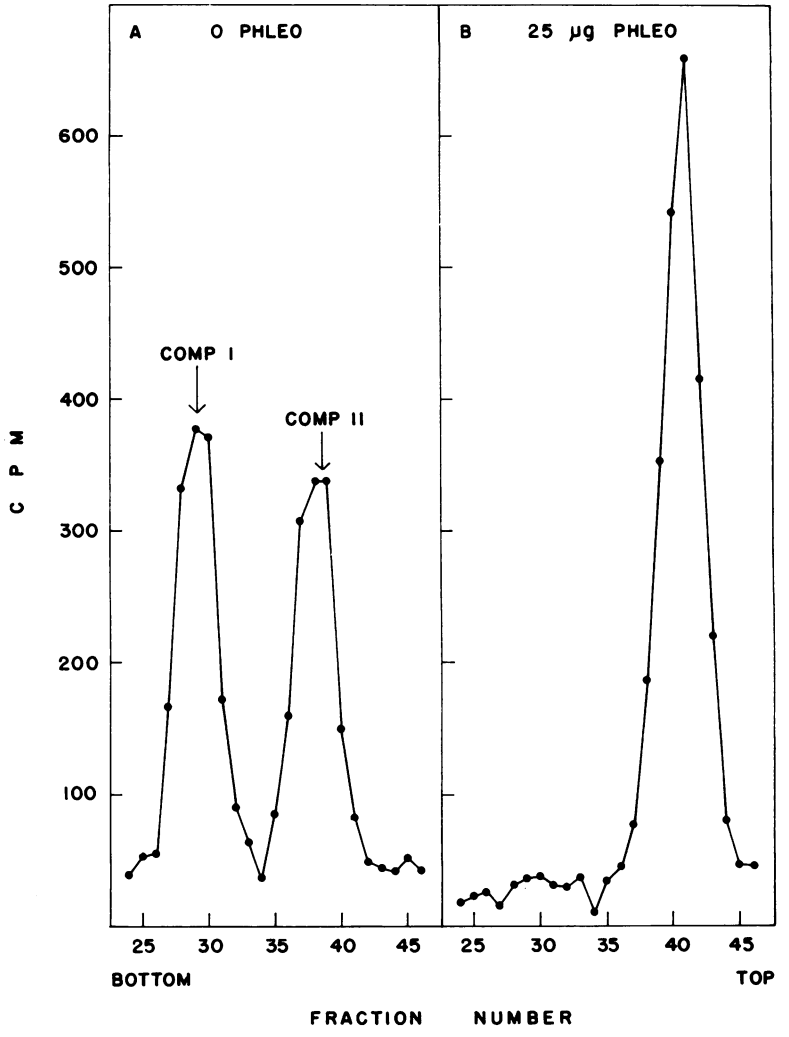

FIG. 5. *Characterization of the DNA from purified virus particles. ^{3}H-thymidine (3 μCi/ml)-labeled complete virus particles from untreated (A) and phleomycin (25 μg/ml)-treated (B) cultures were purified by CsCl density gradient centrifugation and then subjected to alkaline degradation (14). Degraded virus particles were then centrifuged to equilibrium in ethidium bromide-cesium chloride density gradients to separate component I and component II DNA. Conditions for the ethidium bromide-cesium chloride density gradient centrifugation are described in Fig. 3.*

harvested (28 hr postinfection) and DNA was extracted by the Hirt method. A marked increase of ^{14}C prelabel counts in Hirt extracts of phleomycin-treated cultures was observed, and, when the double-labeled DNA from the cultures harvested at 28 hr postinfection was further fractionated by ethidium bromide-cesium chloride centrifugation, it was found that ^{14}C prelabel was not recovered in component I peak in either the untreated (Fig. 8A) or the phleomycin-treated sample (Fig. 8B). However, ^{14}C prelabel counts found in the component II peak were 17% of the ^{3}H postlabel counts for the untreated and 27% for the phleomycin-treated sample. This indicated a possible encapsidation of ^{14}C-labeled host DNA into virus particles, especially in phleomycin-treated cultures. To investigate the possibility of encapsidation of ^{14}C-prelabeled DNA, similarly prepared ^{14}C-prelabeled cultures were infected and postlabeled with ^{3}H-thymidine in the presence or absence of phleomycin, and the virus was purified. Purified polyoma virus thus obtained from either untreated or phleomycin-treated culture did not yield ^{14}C-labeled complete virus particles. Apparently prelabeled and degraded host DNA was not incorporated into virus particles during the

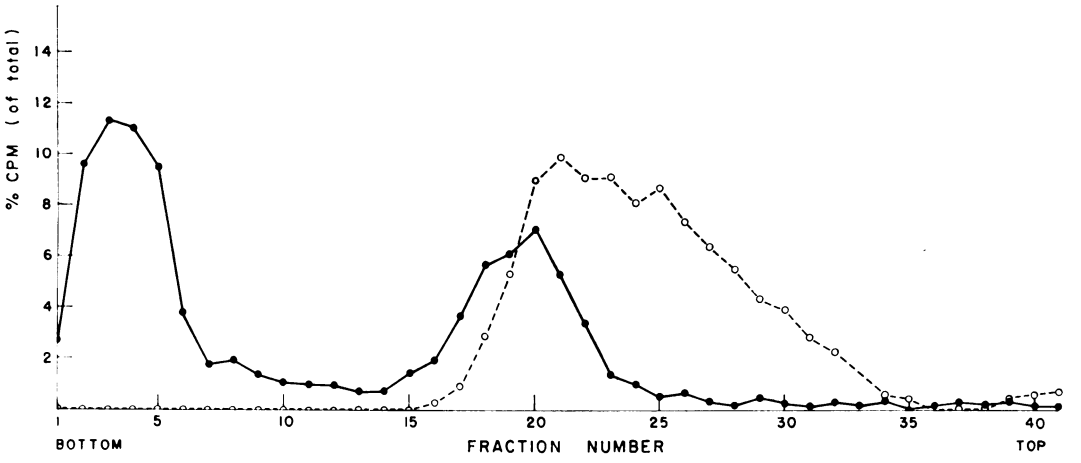

FIG. 6. *Alkaline sucrose density gradient velocity centrifugation of DNA from purified polyoma virus obtained from phleomycin-treated cultures. Viral DNA was isolated as in Fig. 5 and sedimented through an alkaline sucrose gradient (5 to 20% sucrose, 0.1 M NaOH, 0.9 M NaCl; bottom of tube contained a cushion of 0.3 ml of 1.805 g/ml cesium chloride in 20% sucrose) in an SW 50.1 rotor for 4.5 hr at 130,000 × g. Marker polyoma DNA was component I isolated by ethidium bromide-cesium chloride density gradient centrifugation. Component II DNA marker was allowed to form from component I (2 weeks, 4 C). (●) Marker DNA; fractions 1 through 6 contain component I (approximate 53S value); fractions 16 through 23 contain component II (approximate 18S value). (○) DNA from purified polyoma particles obtained from phleomycin-treated cultures.*

TABLE 3. *DNA-DNA hybridization of viral and cellular DNA*

Source of DNA	Total counts/min	Per cent bound to filter[a]		
		Viral	Mouse embryo	No DNA[b]
Component I[c]	1,430	20.56	3.50	2.31
Component II[c]	3,410	6.36	4.70	0.41
Component I + phleomycin[c]	1,220	22.54	1.60	0.32
Component II + phleomycin[c]	2,160	12.36	5.33	1.31
Polyoma particles + phleomycin[d]	2,310	4.50	4.42	0.56
Mouse embryo	5,070	0.26	45.35	0.10

[a] Viral (component I DNA isolated by ethidium bromide-cesium chloride gradient centrifugation) and mouse embryo cell DNA, 6 μg/filter.

[b] Backgrounds not subtracted from per cent bound to filters.

[c] Infected cultures with and without phleomycin (25 μg/ml) were pulsed with ^{3}H-thymidine (10 μCi/ml) from 20 to 25 hr postinfection. DNA was extracted by the Hirt method and the DNA was separated by ethidium bromide-cesium chloride centrifugation.

[d] Polyoma virus was purified from phleomycin (25 μg/ml)-treated cultures, 40 hr postinfection. Viral particles were alkaline-degraded, and the DNA was isolated by ethidium bromide-cesium chloride gradient centrifugation.

40-hr infection regardless of the presence or absence of the antibiotic.

DISCUSSION

Because of its selective inhibitory activity on DNA synthesis, phleomycin has attracted the attention of many investigators in recent years. However, the mechanism of phleomycin inhibition has not been studied extensively. The inhibition of DNA synthesis by phleomycin was thought to be due to its binding at adenine-thymine rich regions of the primer DNA, thus interferring with DNA polymerase activity (7, 16, 17). Tevethia and Rapp (20) reported that phleomycin inhibited synthesis of infectious simian virus 40 in primary green monkey kidney cells. We obtained a similar inhibitory effect (96%) of phleomycin with the polyoma virus-mouse embryo cell system. Earlier findings (7, 19) that phleomycin was a selective inhibitor of DNA synthesis were also confirmed. However, ^{3}H-thymidine incorporation into DNA was completely inhibited in uninfected cells, although phleomycin was a less effective inhibitor in polyoma virus-infected cells. If phleomycin inhibits DNA polymerase by binding DNA, then a competition between phleomycin and DNA polymerase for binding sites on the DNA primer may exist. It has been reported that polyoma infection of mouse kidney cells stimulated activity of cellular DNA-synthesizing en-

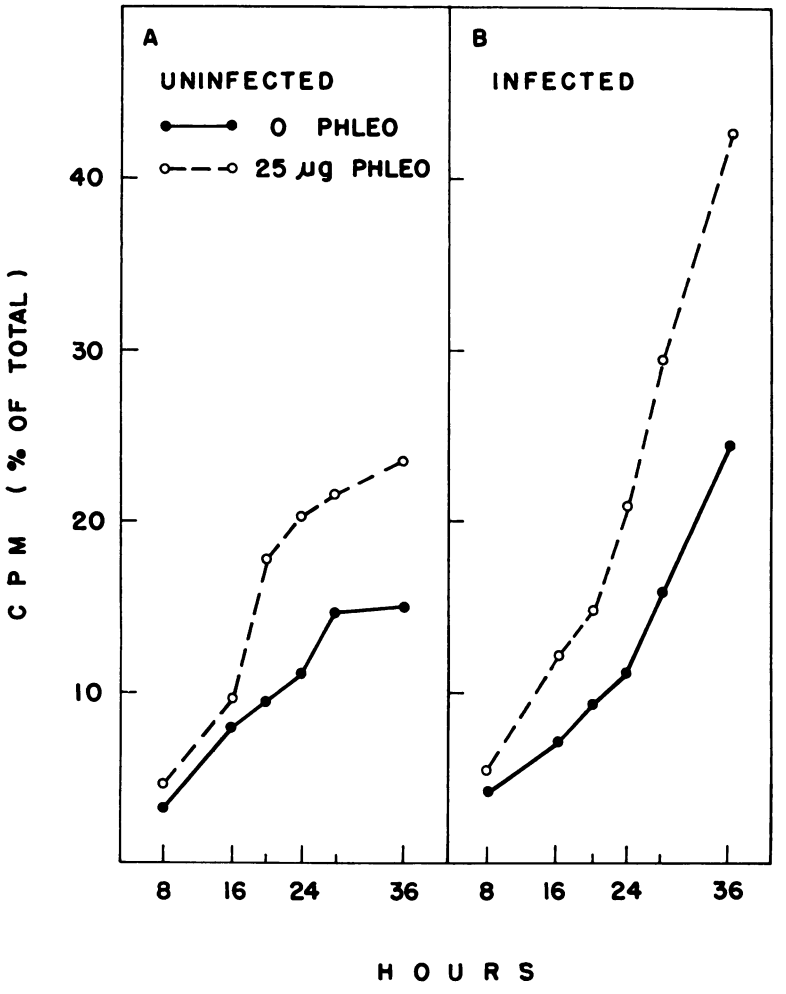

FIG. 7. *Degradation of prelabeled host DNA by phleomycin. Mouse embryo cells were grown in a medium containing ^{3}H-thymidine (1 µCi/ml). Uninfected (A) and infected (B) cultures were maintained in unlabeled medium with or without phleomycin (25 µg/ml), and the medium was collected at the times indicated. Radioactivity was counted for both medium and cells, and per cent (of total) radioactivity appearing in the medium was calculated.*

zymes (5), and possibly the resistance of infected mouse embryo cells against the inhibitory action on ^{3}H-thymidine incorporation could be the result of increased DNA polymerase activity induced by polyoma infection.

Further investigation revealed that polyoma virus antigen (Table 2), polyoma (component I) DNA (Fig. 3), and both complete and incomplete virus particles (Fig. 4) were synthesized in the presence of phleomycin, and these viral components were found in much greater quantities than actual infectivity (Table 1). It was also noted that Hirt extracts from phleomycin-treated cultures always contained supercoiled DNA when DNA was extracted 16 to 28 hr after infection from phleomycin-treated cultures. However, purified polyoma virus from similarly treated cultures, harvested at 40 hr postinfection, lacked circular supercoiled DNA, indicating that supercoiled DNA was nicked and fragmented before packaging.

An extensive DNA breakdown by phleomycin in wild-type *E. coli* cells was reported recently (8). Our results showed that degradation of prelabeled host DNA appeared in the culture medium and in the component II peak after ethidium bromide-cesium chloride gradient centrifugation. However, these host DNA fragments were not encapsidated into virus particles, although encapsidation of host DNA synthesized during viral infec-

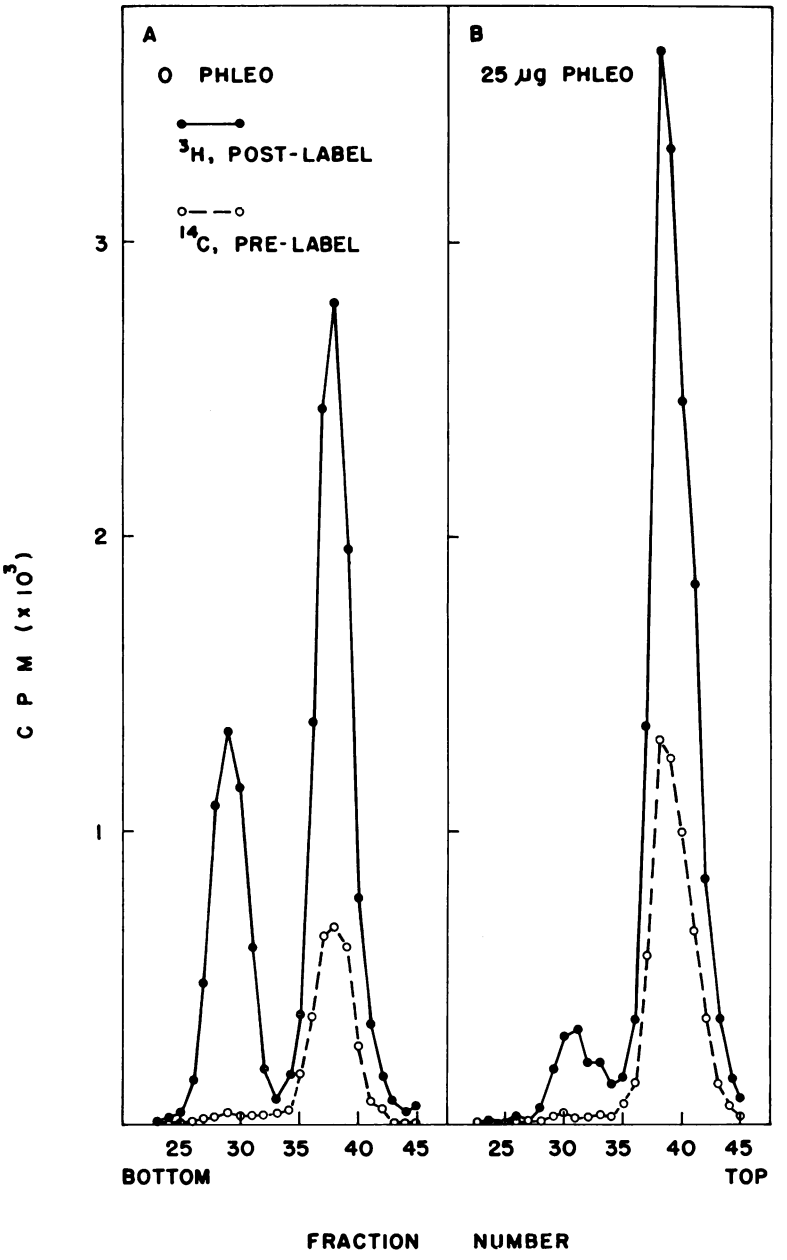

FIG. 8. *Characterization of double-labeled DNA extracted by the Hirt method from infected mouse cells. Mouse embryo cells were prelabeled by growing the cells in ^{14}C-thymidine (0.25 µCi/ml) medium. The ^{14}C-labeled monolayer cultures were infected and pulse-labeled with ^{3}H-thymidine (4 µCi/ml) 26 to 27 hr after infection; DNA was extracted by the Hirt method (9) at 28 hr postinfection. The DNA was further separated into component I and component II DNA by ethidium bromide-cesium density gradient centrifugation as described in Fig. 3.*

tion did occur (Table 3). There was no suggestion from in vitro studies (7) that phleomycin induced breaks in DNA directly. It is possible that the binding of phleomycin to the DNA primer could be providing target sites for cellular endonuclease, since in the culture of *E. coli* mutant lacking endonuclease phleomycin did not cause fragmentation of cellular DNA (8).

The results reported here demonstrated that phleomycin allowed viral protein and viral DNA synthesis but inhibited synthesis of infectious virus particles and that the cause of noninfectivity appeared to reside in defective DNA contained in these particles. The absence of supercoiled DNA from such particles suggested the following possibilities: (i) viral DNA was made in smaller pieces; (ii) circularization of polyoma DNA by ligase was not operating; or (iii) circular supercoiled DNA was made but fragmented before packaging. However, it is more likely, since ^{3}H-thymidine was incorporated into DNA and component I was made in phleomycin-treated cultures, that both DNA polymerase and ligase were functioning. In addition, fragmentation of the viral DNA was observed (Fig. 6), and an increased rate of prelabeled host DNA degradation indicated an enhanced cellular nuclease activity.

The process of DNA replication requires three enzymatic actions: in addition to DNA polymerase, nickase and ligase are necessary to initiate and complete DNA replication (1). It is likely that effective inhibitory activity of phleomycin on the synthesis of biologically active DNA could be the sum of phleomycin effects on all three enzymes involved in DNA replication. The effect of phleomycin on DNA polymerase, ligase, and nucleases in the polyoma-mouse cell system is currently being investigated in this laboratory.

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