Precursor Products Found in Formaldehyde-fixed Lysates of BHK-21 Cells Infected with Pseudorabies Virus

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Lysates of BHK-21 cells, infected with pseudorabies and labeled with ^3H -thymidine, were treated with formaldehyde and centrifuged in preformed CsCl gradients. Five peaks, designated bands A to E, were detected and shown to contain the following virus-specific products: band A, aggregates of virions; band B, virions; band C, empty capsids and nucleocapsids; bands D and E, small nucleoid-like particles. All five bands contained viral deoxyribonucleic acid and virus-specific antigens. Deoxyribonuclease treatment of the lysates before centrifugation resulted in the complete loss of ^3H -thymidine-labeled material from bands D and E; bands B and C were unaffected. Pulse-chase studies showed that ^3H -thymidine can be rapidly chased into the virus-specific products sedimenting in bands D and E. Further chase resulted in the gradual loss of label from bands D and E with a concomitant increase in the amount of label in bands A, B, and C. These results indicate that the virus-specific products in bands D and E are precursors of pseudorabies virions and may represent the nucleoid of the virion.

The complete virion of herpesvirus is made up of three major components: the inner core or nucleoid, the protein capsid, and the outer lipoprotein envelope (1, 6, 13). The nucleoid has been shown to measure between 50 and 75 nm in diameter in thin sections of infected cells (6, 12, 23). A variety of evidence, including staining characteristics (21), measurement of radiation-sensitive volume (14, 15), and size discrepancies (23), indicates that the nucleoid must contain other material besides deoxyribonucleic acid (DNA; 6). The nucleoid together with the protein capsid compose the nucleocapsid particle that is 110 nm in diameter. The nucleocapsid is surrounded by a lipoprotein envelope partially derived from host membranes but also containing virus-specific antigens (1, 5, 17, 21). This enveloped structure comprises the complete infectious virion which measures approximately 180 nm in diameter.

Spring and Roizman (19) have reported that herpes simplex virus is degraded upon centrifugation in CsCl . They found that the virion and other viral products previously not found during centrifugation of infected cell lysates could be detected by fixation of the lysates with formaldehyde before centrifugation. These products were identified (20) as 110-nm nucleocapsids

and small particles 25 to 35 nm in diameter. It became of interest, therefore, to determine the origin and fate of these products in light of the following two hypotheses: these products are artifacts caused by the degradation of complete virions in CsCl even after stabilization with formaldehyde, or these products represent precursors of the complete virion.

MATERIALS AND METHODS

Solutions. TEA consisted of 0.02 M triethanolamine plus 0.05 M KCl and 0.001 M MgCl_2 at a pH of 7.08 (19). TEA-F consisted of TEA buffer containing 28% formaldehyde at a pH of 7.08. NaCl buffer consisted of 0.15 M NaCl, 0.07 M Na_2HPO_4 , and 0.03 M NaH_2PO_4 . SCS consisted of saline-citrate solution as described by Marmur (11). PBS-G consisted of phosphate-buffered saline prepared according to Dulbecco and Vogt (3) containing 0.1% glucose and 1% fetal calf serum.

Cells. Starter cultures of BHK-21 cells were obtained from Microbiological Associates, Inc. They have been carried in this laboratory in monolayer cultures by using minimal essential medium (MEM) of Eagle (4) containing 5% fetal calf serum as growth medium. Maintenance medium was MEM plus 1% fetal calf serum.

Virus. The strain of pseudorabies virus (PSV) used in this study was originally obtained from E. R. Pfefferkorn. Plaque assay of the virus was performed

in BHK-21 cells in a maintenance medium overlay containing 1% methyl cellulose. After incubation for 40 hr at 37 C, the cell sheet was fixed with ethyl alcohol and stained with Giemsa stain, and the plaques were counted.

Radiochemicals. The following radiochemicals were purchased from New England Nuclear Corp. (Boston, Mass.): thymidine-*methyl*- ^3H (specific activity, 6.7 c/mm), thymidine-2- ^{14}C (25 mc/mm), ^{14}C -L-leucine (250 mc/mm), and ^{14}C -L-arginine (220 mc/mm).

Preparation and centrifugation of formaldehyde-fixed cell lysates. Monolayer cultures of BHK-21 cells in 32-oz (0.946-liter) bottles were infected with PSV at a multiplicity of 50 plaque-forming units (PFU)/cell, and the bottles were shaken gently for 2 hr at 37 C to facilitate adsorption. The inoculum was aspirated, maintenance medium containing radioactive ^3H -thymidine was added, and the bottles were incubated at 37 C for various periods as described in Results. The cells were scraped, centrifuged, washed with PBS-G and recentrifuged. The cell pellet was resuspended in 1 ml of distilled water, and the cells were allowed to swell for 5 min. The cells were then disrupted by 20 to 30 strokes with a tight-fitting Dounce homogenizer or in a Raytheon Sonic Oscillator at maximum frequency for 15 sec. Both methods of cell disruption were equally effective and resulted in similar patterns when the lysates were centrifuged in CsCl. After disruption, the cell debris was removed by centrifugation at $800 \times g$ for 30 min. The lysates were adjusted to 0.02 M TEA by addition of 0.1 M TEA buffer; TEA-F was added to give a final concentration of 3% formaldehyde. The fixed lysates were kept at 4 C for 16 to 24 hr before centrifugation in CsCl. Preformed CsCl gradients were prepared in SW41 centrifuge tubes by using a two-chambered gradient maker. The two original concentrations of CsCl used were 1.175 and 1.575 g/cc. A 0.5- to 1.0-ml sample of each lysate was layered onto the gradient, and the tubes were centrifuged at $210,000 \times g$ for 20 to 24 hr at 15 C. After centrifugation, the bottom of the tubes was punctured and four-drop fractions were collected directly into tubes containing 1.0 ml of SCS. At every 15th fraction, one drop was placed in a Bausch & Lomb refractometer for determination of the refractive index. Trichloroacetic acid was added to each tube to a final concentration of 5%. The precipitates formed were collected on type HA membrane filters (Millipore Corp., Bedford, Mass.), washed with 5% trichloroacetic acid and 70% ethyl alcohol, and air-dried. The filters were immersed in 5 ml of toluene-base scintillation fluid for determination of acid-precipitable radioactivity in a Packard liquid scintillation spectrometer.

Preparation of PSV antigen (PSV-Ag). A large pool of PSV with a titer of 7×10^8 PFU/ml was prepared in BHK-21 cells and used as the antigen source. The antigen was prepared from the virus pool by the method of Roizman et al. (18). Briefly, the pH of the pool was adjusted to 1.0 with 1 N HCl, and the pool was incubated at 37 C for 1.5 hr and 4 C for 16 hr. It was then placed in a boiling-water bath for 1.5 hr and centrifuged, and the supernatant fluid (PSV-Ag) was stored at 4 C. This procedure results in the degra-

dation of PSV virions to produce a variety of antigenic determinants. An undiluted sample of this antigen preparation produced no cytopathogenic effects when inoculated into a monolayer of BHK-21 cells.

Preparation of PSV antibody (PSV-Ab). Rabbits were inoculated in the footpads with PSV-Ag mixed with Freund adjuvant. Starting 3 days later, intraperitoneal injections of PSV-Ag were given daily to each rabbit for 22 days at which time the rabbits were test bled, and their sera were titrated for neutralizing antibody by a plaque-inhibition test. The animals were exsanguinated, the high-titered sera were collected, pooled, and inactivated at 50 C for 30 min. To eliminate the antibodies against host-cell antigens, the serum was mixed with 1 ml of packed, disrupted BHK-21 cells and shaken at 37 C for 1 hr. This step was repeated with fresh cells two times, after which the serum was centrifuged at $25,000 \times g$ for 2 hr. The supernatant fluid (PSV-Ab) was removed and stored at -20 C.

Indirect precipitation test. The indirect precipitation test was based on the method described by Hamada and Kaplan (7). Four monolayer cultures of BHK-21 cells were either mock-infected or infected with 50 PFU/cell of PSV. The cells were incubated for 22 hr at 37 C in maintenance medium containing 20 μC of ^3H -thymidine, 10 μC of ^{14}C -leucine, and 10 μC of ^{14}C -arginine. Formaldehyde-fixed lysates were prepared and centrifuged as described; 0.1-ml samples from each fraction were precipitated with 5% trichloroacetic acid, and the amount of radioactivity was determined. The remainder of each fraction was dialyzed against NaCl buffer and divided into two 0.5-ml portions labeled A and B. An 0.1-ml sample of PSV-Ab was added to tube A, and 0.1 ml of buffer was added to tube B. After incubation of both tubes at 37 C for 2 hr, 0.2 ml of sheep anti-rabbit serum was added to tube A and 0.2 ml of buffer was added to tube B. Both tubes again were incubated at 37 C for 2 hr and then at 4 C overnight. The tubes were centrifuged for 20 min at $800 \times g$, and the supernatant fluid was decanted. The precipitates were resuspended in NaCl buffer and collected on membrane filters (Millipore Corp.) which then were rinsed with NaCl buffer and air-dried. The filters were placed in vials, toluene-base scintillation fluid was added, and the radioactivity was determined.

DNA extraction and analysis. Monolayer cultures of BHK-21 cells were either infected or mock-infected, and 20 μC of ^3H -thymidine in maintenance medium was added to each culture after 2 hr. At 18 hr after infection, formaldehyde-fixed lysates were prepared and centrifuged as described. Peak fractions from each band were removed and dialyzed against NaCl buffer; the DNA was extracted by the method of Roizman and Roane (16). The extracted DNA was mixed with CsCl and centrifuged in an SW39 rotor at $140,000 \times g$ for 44 hr at 15 C. Five-drop fractions were collected and one drop was used for a refractive index reading after every fifth fraction. The fractions were precipitated with 5% trichloroacetic acid, and the radioactivity in the precipitates was determined.

Deoxyribonuclease test. Uninfected and PSV-infected BHK-21 cells, labeled with ^3H -thymidine, were

scraped at 16 hr after infection and suspended in 1 ml of distilled water as described. $MgCl_2$ was added to each fraction to a final concentration of 10^{-2} M, and each fraction was divided into two equal samples. Deoxyribonuclease (100 μ g per 0.1 ml of sample) was added to one sample and distilled water to the other. All samples were incubated at 37 C for 30 min, rapidly chilled, and fixed with 3% formaldehyde. After 16 hr at 4 C, each sample was centrifuged in a CsCl gradient at 4 C, and fractions were collected and analyzed as described.

RESULTS

Effect of formaldehyde fixation on stability of of PSV. Preliminary studies revealed that the infectivity of PSV was reduced by 97% upon centrifugation in CsCl. The following experiment was done to determine the effect of formaldehyde on the stabilization of PSV products during centrifugation in CsCl. PSV-infected or mock-infected BHK-21 cells, labeled with 20 μ C of 3H -thymidine, were extracted after 18 hr of incubation and suspended in distilled water as described. Each sample was then divided into two equal portions. One portion was treated with TEA only, and the other was fixed with TEA-F to a final concentration of 3% formaldehyde. All four samples were centrifuged and collected as described (Fig. 1). The unfixed sample of infected cells showed two peaks or bands of acid-precipitable material; one at a density of 1.28 g/cc and a small band at 1.20 g/cc. Formaldehyde fixation of infected cell lysates resulted in the stabilization of virions, as noted by the four-fold increase in radioactivity in the band at 1.28 g/cc (band B). The presence of three other bands of acid-precipitable material not present in unfixed samples was also noted in the formal-

dehyde-fixed samples. The five bands found in these formaldehyde-fixed preparations of infected cells have been designated bands A, B, C, D, and E, with the following buoyant density ranges: band A, 1.20 g/cc; band B, 1.28 g/cc; band C, 1.325 to 1.350 g/cc; band D, 1.40 to 1.45 g/cc; and band E, 1.50 to 1.55 g/cc. As seen in Fig. 1, uninfected, unfixed cells showed no acid-precipitable bands upon centrifugation. In formaldehyde-fixed, uninfected cells, a single band at a density of 1.40 g/cc was found which probably consists of stabilized host nucleoprotein material.

Identification of host material. Since it was shown above that formaldehyde-fixed preparations of uninfected cells have a band at a density of 1.40 g/cc, and since this band roughly corresponds to band D in infected cells, it was evident that some of the material in band D must consist of host material. The following experiment was performed to determine the extent of host-cell contamination and the fate of host material upon infection. Monolayers of BHK-21 cells were incubated in growth medium containing 25 μ C of ^{14}C -thymidine. After 24 hr, the medium was removed, and the cells were washed three times with warm PBS-G. Growth medium containing a 100-fold excess of "cold" thymidine was added and the cells were incubated for 24 hr. The cells were then either infected or mock-infected, maintenance medium containing 20 μ C of 3H -thymidine was added, and the cells were incubated for 18 hr. Formaldehyde-fixed lysates were prepared and centrifuged as described (Fig. 2). Both the amount and the distribution of ^{14}C -thymidine-labeled host material

FIG. 1. Effect of formaldehyde fixation of 3H -thymidine-labeled infected and uninfected cell lysates upon centrifugation in preformed CsCl gradients. The five bands formed upon centrifugation of formaldehyde-fixed infected cell lysates are designated bands A, B, C, D, and E. Fraction 1 is the bottom of the gradient.

FIG. 2. Distribution of host cell material labeled before infection in infected and uninfected cell lysates. Cells were labeled with ^{14}C -thymidine before infection and then infected or mock-infected and labeled with 3H -thymidine. At 18 hr after infection, formaldehyde-fixed lysates were prepared and centrifuged in preformed CsCl gradients as described. (A) Infected cell lysates; (B) uninfected cell lysates. Symbols: $\circ$ 3H ; $\bullet$ ^{14}C .

were the same in lysates from both uninfected and infected cells. This indicates that host DNA is not degraded during infection and redistributed to bands A, B, C, and E. The following series of experiments show that host material does not account for all of the products in band D.

Identification of the products contained in the bands by electron microscopy. Cells were infected or mock-infected and labeled with 30 μ Ci of 3 H-thymidine after 2 hr of adsorption. At 18 hr after infection, formaldehyde-fixed lysates were prepared and centrifuged in CsCl as described, and peak fractions were removed from the five infected cell bands (A to E). In the mock-infected sample, peak fractions were collected from the uninfected cell band as well as from fractions corresponding in density to bands A, B, C, and E of infected cells. The samples were dialyzed against distilled water, placed on grids, stained with potassium phosphotungstate (pH 6.8 to 7.2), and examined with an electron microscope (model B6 AEI). No samples taken from the mock-infected cell fractions contained any morphological structures similar to those in the infected cell bands. The morphology of the products found in the five infected cell bands is shown in Fig. 3. Band A contained aggregates of complete virions attached to membrane material (Fig. 3a) and large aggregates containing complete virions, empty capsids, and nucleocapsids which are not shown. Band B contained complete virions measuring 180 to 200 nm in diameter. The envelopes of some of the virions showed some distortion (Fig. 3b) but most contained completely intact envelopes (Fig. 3c). Band C contained primarily empty capsids but also contained some nucleocapsid particles. Both particles measured approximately 110 to 115 nm in diameter. The small particles shown in Fig. 3e were found in both bands D and E. These particles varied in size from 45 to 79 nm in diameter but appeared morphologically identical. Whether size differences alone can account for the separation of these particles into the two bands cannot be ascertained at this time. Deoxyribonuclease treatment of bands D and E after centrifugation resulted in a significant loss of these particles, suggesting that they represent the deoxyribonuclease-sensitive material noted in Table 2.

DNA extraction. DNA from bands A to E and the uninfected cell band was extracted and centrifuged as described above. As controls, DNA was extracted directly from 18-hr infected and uninfected BHK-21 cells labeled with 50 μ Ci of 3 H-thymidine. The controls showed that PSV DNA had a density of 1.730 to 1.732 g/cc, and BHK-21 cell DNA had a density of 1.680 to 1.685 g/cc. The results of DNA extraction from

infected and uninfected cell bands after CsCl centrifugation are shown in Table 1. Viral DNA was found in all five infected cell bands. As would be expected from the previous results (Fig. 2), host-cell DNA was also found in band D. The host DNA in band C was probably contamination from band D.

Deoxyribonuclease treatment. The following experiment was performed to determine whether the 3 H-thymidine-labeled material in infected and uninfected cell bands was susceptible to deoxyribonuclease. The procedures for extraction and analysis are described above, and the results are summarized in Table 2. Material in bands B and C were not sensitive to deoxyribonuclease treatment. Material in bands D and E and material in the uninfected band were completely lost by deoxyribonuclease treatment. The counts lost from bands D and E after deoxyribonuclease treatment accumulated at the top of the gradient completely masking the counts in band A.

Indirect precipitation test. The immunological specificity of the material in the bands formed upon centrifugation of formaldehyde-fixed lysates of uninfected and infected cells was determined by the following experiment. The procedures have been described above, and the results of one such study are summarized in Fig. 4. Although the cells had been labeled with both 14 C-arginine and 14 C-leucine as well as with 3 H-thymidine, only the 3 H-thymidine patterns have been plotted to simplify the graph. The results of the indirect precipitation test, plotted as a histogram, were calculated by subtracting the amount of radioactivity precipitated in tube B (buffer only) from the amount precipitated in tube A (PSV-Ab plus sheep anti-rabbit serum). Some radioactive antigens were precipitated in the uninfected cell band, indicating that all of the host-cell antibodies were adsorbed from the PSV-Ab by the adsorption procedure used. It is possible, therefore, that a small per cent of the total antigens precipitated in the infected cell sample would be of host origin. However, it can be readily seen from the results of the infected cell sample that all five infected bands contained viral-specific antigens.

3 H-thymidine uptake during infection. During a continuous 18-hr labeling period, 3 H-thymidine became incorporated into all five infected cell bands (Fig. 1). Therefore, it became of interest to determine the distribution of 3 H-thymidine in these bands at various times after infection. Monolayer cultures of BHK-21 cells were infected or mock-infected, and 20 μ Ci of 3 H-thymidine per bottle was added after a 2-hr adsorption period. At 3- to 6-hr intervals, formaldehyde-

FIG. 3. Electron micrographs of pseudorabies virions and virus-specific products isolated from formaldehyde-fixed lysates of infected cells. Peak fractions from the five infected cell bands present after centrifugation of the lysate in CsCl (see Fig. 1) were dialyzed against distilled water, stained with potassium phosphotungstate, and examined. (a) Membrane-bound aggregates of virions from band A; (b) virion from band A with a distorted envelope; (c) virion from band B with the envelope intact; (d) empty capsids and nucleocapsids from band C; (e) nucleoid-like particles seen in bands D and E. (a) $\times 200,000$; (b, c, d, and e), $\times 250,000$.

fixed lysates were prepared and analyzed as described. The total acid-precipitable counts in each band were calculated for each time interval, and the data were plotted as shown in Fig. 5. The accumulation of acid-precipitable counts into band B (virions) shows good correlation with one-step growth curves of PSV in BHK-21 cells (*unpublished data*); i.e., intracellular virus continues to increase until 16 hr after infection. Acid-precipitable label did not accumulate to any extent in nucleocapsids (band C) during the course of the infection, suggesting that soon after the nucleocapsids are formed they acquire an envelope. The salient feature of this study is the pattern of acid-precipitable material in bands D and E. Label accumulated in these two bands through 10 hr after infection and then began to decline. The loss of label from these bands and the continued uptake of label into bands A and B suggest that the products in bands D and E are being incorporated into virions. If the material in bands D and E was a result of degradation of virions, one would expect that the amount of label present in these two bands would increase in proportion to the amount of label in the infectious virion band during the course of the infection. That this is not the case suggests that these bands are not degradation products. However, from the patterns shown in Fig. 3, it cannot be stated *a priori* that the products in bands D and E are precursors of infectious virions. However, evidence that supports this hypothesis is shown in the next series of experiments.

Distribution of label in bands during a pulse-

TABLE 1. Identification of the DNA extracted from infected and uninfected cell bands^a

Type of DNA	Density (g/cc)	Band					
		A	B	C	D	E	Uninfected cells
Host.....	1.680	0	0	+	+	0	+
Viral.....	1.730	+	+	+	+	+	0

^a Symbols: +, DNA present; 0, DNA absent.

chase. Since the results of the preceding study indicated that the products formed in bands D and E were not degradation products or artifacts of fixation, it became of interest to study the kinetics of ³H-thymidine uptake into these bands. Hamada and Kaplan (7) showed that host DNA synthesis is completely shut off by 7 hr after infection with PSV. Therefore, the following experiment was performed by using cells 8 hr after infection to reduce contamination of the bands by labeled host DNA. Monolayer cultures of BHK-21 cells (2.8×10^8 cells) were infected or mock-infected and incubated at 37 C for 8 hr. The cells were scraped, centrifuged, and resuspended in 10 ml of maintenance medium containing 300 μ c of ³H-thymidine. After incubation at 37 C for 5 min, the cells were centrifuged and resuspended in 500 ml of maintenance medium containing a 100-fold excess of "cold" thymidine. The cells were kept in suspension in a water bath at 37 C, samples were removed at various chase times and poured over a frozen slurry of PBS-G to stop incorporation, and lysates were prepared and analyzed as described. The total counts of acid-precipitable radioactivity present in each band were calculated for uninfected and infected cells. Uninfected cells showed a linear increase of ³H-thymidine uptake into the uninfected band during the 2-hr chase period. In infected cells (Fig. 6), label was rapidly incorporated into bands D and E through a 30-min chase period. After 30 min, the amount of label in these bands began to decline with a concomitant increase in the amount of label chased into bands A, B, and C. The kinetic pattern observed in this study supports the hypothesis that the products formed in bands D and E are precursors of complete virions.

DISCUSSION

This paper has shown that the virions of PSV and smaller virus-specific products identified as empty capsids, nucleocapsids, and nucleoid-like particles can be detected by treatment of the infected cell lysate with formaldehyde before centrifugation in CsCl gradients. This finding is in agreement with previous studies with herpes

TABLE 2. Effect of deoxyribonuclease on the bands from uninfected and infected cell lysates

Determination	Total counts per min per band					
	E	D	C	B	A	Uninfected
No deoxyribonuclease.....	5,271	6,824	2,120	9,872	3,130	12,110
With deoxyribonuclease.....	28	212	1,979	9,950	— ^a	128

^a The counts/min in the A band could not be determined because of masking by an increase in counts/min at the top of the gradient, lost from the E and D bands after deoxyribonuclease treatment.

FIG. 4. Distribution of labeled antigens precipitated by an indirect precipitation test by using PSV-Ab plus anti-rabbit sheep antibody. Cells were infected or mock-infected and labeled for 16 hr with ^3H -thymidine and ^{14}C -leucine plus ^{14}C -arginine. Formaldehyde-fixed lysates were prepared and centrifuged in CsCl gradients as described. Fractions were collected, and 0.1-ml samples were precipitated with 5% trichloroacetic acid to obtain the distribution pattern of the bands (●). To simplify the graph, only the counts obtained from ^3H -thymidine label were plotted to orient the fractions. The remainder of each fraction was dialyzed against NaCl buffer and precipitated with PSV-Ab plus anti-rabbit sheep antibody. The amount of radioactivity-labeled antigens precipitated from each fraction was plotted as a histogram.

simplex virus (19, 20) and indicates the usefulness of formaldehyde in the study of herpesviruses. The products in the five infected cell bands have been shown to be virus-specific by extraction and analysis of the labeled DNA from each band and by precipitation of labeled antigens from each band with virus-specific antiserum. The salient findings of this study are the results obtained in the pulse-chase experiments which indicate that the viral structures (nucleoid-like particles) in bands D and E are precursors of the complete virion and not products of degradation or artifacts of formaldehyde fixation.

The conclusion that the nucleoid-like particles in bands D and E represent precursors is based on two series of experiments. In the first series (Fig. 5), the distribution of ^3H -thymidine in the five infected cell bands during a continuous labeling period was determined. In these experiments, the uptake of ^3H -thymidine into the five infected cell bands is a measure of the accumulation of DNA into these bands during a 22-hr infectious cycle, and not of the rate of DNA synthesis. Progeny virus can be found in cells as early as 4 hr after infection, as evidenced by a small amount of radioactivity in band B. DNA continues to accumulate in bands D and E until 10 hr after infection, indicating that by this time viral DNA synthesis is complete. This ob-

FIG. 5. Total uptake of ^3H -thymidine-labeled, acid-precipitable material in the uninfected and infected cell bands after centrifugation in CsCl gradients. Cells were infected or mock-infected and ^3H -thymidine was added after 2 hr. Samples were taken at various times after infection, and formaldehyde-fixed lysates were prepared and centrifuged in CsCl gradients. The total counts/min incorporated in each band were calculated and plotted against time.

FIG. 6. Distribution of ^3H -thymidine label into acid-precipitable material in a 2-hr pulse-chase study. Cells were infected and, at 8 hr after infection, pulsed for 5 min with ^3H -thymidine. The cells were suspended in a chase medium, and samples were removed at various times through 2 hr. Formaldehyde-fixed lysates were prepared and centrifuged in CsCl as described, and the total counts/min in each band were calculated and plotted against the chase time.

servation is in accord with previous studies of the kinetics of DNA synthesis in PSV-infected cells by Kaplan and Ben-Porat (8). The fact that no appreciable uptake of DNA into band C could be observed suggests that the nucleocapsid particles are rapidly enveloped and do not accumulate to any extent during the infectious cycle. Since it was shown previously (2) that only about 12% of the total viral DNA synthesized becomes incorporated into virions, it is not surprising to find excess viral products in bands C, D, and E as late as 22 hr after infection.

In the second series of experiments, the results of a pulse-chase study (Fig. 6) showed that, after a 5-min pulse, ^3H -thymidine is rapidly incorporated into bands D and E during the first 30 min of chase. Label then is lost from these bands with a concomitant increase of label into bands A, B, and C. These kinetics indicate that the material in bands D and E consists of precursor structures which become incorporated into larger, less dense particles (i.e., nucleocapsids and virions) upon prolonged incubation. The rate of accumulation of counts into bands D and E is faster than the rate of loss of counts after a 30-min chase. This suggests that these nucleoid-like structures are being synthesized in excess, forming a "pool" from which they are withdrawn to be incorporated into virions.

There is good evidence that the nucleoid of herpesviruses must contain other material besides DNA (14, 15, 21, 23). This complex, probably consisting of DNA plus internal proteins of the virions, may exist as a structural entity upon which are assembled the capsomeres to form the nucleocapsid particle. Several lines of evidence presented in this paper support the hypothesis that the virus-specific particles found in bands D and E may represent the nucleoid of the virion. First, the size of the particles in bands D and E (45 to 79 nm) is in direct accord with previous thin-section measurements of the nucleoid (6, 12, 23). The internal diameter of empty capsids in band C measured approximately 90 nm in diameter, indicating that the nucleoid-like particles have the proper physical dimensions to "fit" inside the hollow cavity of the capsid. Second, electron microscopy revealed a significant loss of nucleoid-like particles after deoxyribonuclease treatment. This result parallels the finding that deoxyribonuclease treatment of infected cell lysates resulted in a significant decrease in the radioactive material recoverable in bands D and E after CsCl centrifugation (Table 2). Third, it has also been observed that structures similar to those seen in bands D and E can be recognized in unfixed and fixed preparations of infected cells before fractionation in CsCl gradients. Thus, the

kinetic evidence and morphological evidence presented in this paper support the hypothesis that the nucleoid-like particles in bands D and E are precursors of virions and are not degradation products of virions or artifacts caused by formaldehyde fixation or centrifugation in CsCl.

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