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Herpes Simplex Virus Products in Productive and Abortive Infection

I. Stabilization with Formaldehyde and Preliminary Analyses by Isopycnic Centrifugation in CsCl

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Lysates of HEp-2 cells productively infected with herpes simplex virus yielded two bands on isopycnic centrifugation in CsCl gradients, ranging from 1.2 to 1.6 g/cm³. One band, designated α, had a mean buoyant density of 1.27 g/cm³ and contained herpes virions. Band β had a mean density of 1.305 g/cm³ and contained primarily complement-fixing viral antigens and little or no viral deoxyribonucleic acid (DNA). The products banding in the α and β bands were unstable; fivefold or higher amounts were recovered by treating the cell extract with formaldehyde prior to centrifugation. Formaldehyde treatment increased the buoyant density of viral products in both the α and β bands by about 0.015 g/cm³. In addition, it stabilized hitherto inapparent products, forming a broad band γ with a density range of 1.37 to 1.45 g/cm³. The material in the γ band was heterogeneous; it contained viral DNA, cellular DNA, and viral antigen. Formalinized lysates of DK cells abortively infected with herpes simplex virus yielded a β band undifferentiated from that formed by extracts of productively infected cells. The γ band was less dense and narrower. The α band was entirely missing.

Previous reports (1, 2, 10) have shown that strain MP*dk*⁻ of herpes simplex virus multiples in human (HEp-2) cells but not in dog kidney (DK) cells. The abortive infection of DK cells is characterized by the following: (i) at low [$\leq$10 polykaryocyte-forming units (PoFU)/cell] multiplicities of infection, only interferon is produced; (ii) at relatively high multiplicities of infection ($>$100 PoFU/cell), viral antigen and deoxyribonucleic acid (DNA) are produced, but particles with physical characteristics of herpes virions are not assembled; (iii) the immediate cause of abortive infection appears to be the absence of one or more functional proteins making up the virion.

It is obvious that, to understand the cause of the failure of MP*dk*⁻ to multiply in DK cells, one must ascertain what viral proteins are nonfunctional or lacking in the abortively infected cells. Experimentally, the question we have asked is whether the DNA, and other constituents of the herpes virion made in abortive infection, aggregate, and, if this is the case, what are the properties of the aggregate. In these experiments, we centrifuged lysates of productively and abortively infected cells to equilibrium in CsCl density gradients. We then traced the position of DNA by labeling it with thymidine and that of viral antigen by complement fixation with suitable antisera. It is noteworthy that the results of initial experiments were unsatisfactory for two reasons. First, extracts from 10^9 infected DK cells per tube were needed to obtain measurable bands of labeled DNA and viral antigen. Second, the position of the bands was erratic and unpredictable. The basis for these difficulties emerged when it was discovered that the herpes virion is unstable in CsCl solution. It seemed probable that, if the virion is unstable, the DNA-protein aggregate forming the precursors should be even less stable.

We should point out that previous work from this laboratory (13) reported relatively good recovery of infectivity from CsCl gradients containing excess bovine albumin. This procedure to stabilize the virus was a priori unsatisfactory for the aims of this work. The purpose of this paper is (i) to document the instability of the herpes virion in CsCl density gradients, (ii) to show that the virion can be stabilized by treatment with formaldehyde prior to centrifugation, and (iii) to furnish the results of isopycnic centrifugation in CsCl solutions of stabilized lysates of productively and abortively infected cells. Formaldehyde was used as a stabilizing agent for two reasons. First, it is an excellent fixative for preparing virus

for examination by electron microscopy. Second, formaldehyde has been successfully used by Perry and Kelley (9) to stabilize ribosomes for centrifugation in CsCl density gradients.

MATERIALS AND METHODS

Solutions. PBS-A-G is phosphate-buffered saline made according to Dulbecco and Vogt (3), but containing 0.2% bovine albumin (Fraction V, Armour Laboratories, Chicago, Ill.) and 1% glucose. SCS is a saline-citrate solution prepared as described by Marmur (7). TEA is 0.02 M triethanolamine containing 0.05 M KCl and 0.001 M MgCl buffered at *p*H 7.05 or at 7.75 by the addition of HCl. Formaldehyde was a 28% stock solution made in TEA buffer. Veronal buffer was made according to the method of Osler et al. (8).

Radiochemicals. Thymidine-methyl-^{3}H (specific activity 18 c/mmole) was obtained from Nuclear Chicago Corp., Chicago, Ill.

Cesium chloride. The cesium chloride grades used in these studies were (i) a grade designated 99.9% pure obtained from A. D. Mackay Inc., New York, N.Y., (ii) an optical grade obtained from S. H. Cohen, Yonkers, N.Y., and (iii) an optical grade designated as "0.06" obtained from Gallard Schlesinger Co., Garden City, N.Y. The CsCl solutions were filtered to remove insoluble particulate matter before use. There were no appreciable differences in the results obtained with the various grades of CsCl.

Cells. The HEp-2 cell line was originally obtained from Microbiological Associates, Inc., Bethesda, Md. The continuous line of dog kidney (DK) cells (originally obtained from M. D. Hoggan, Laboratories of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health) has been carried in our laboratory since 1963. The pertinent properties of these cell lines have been described (1, 10).

Antibody. The rabbit convalescent serum used for complement-fixation tests was the kind gift of Albert B. Sabin. The serum was obtained from a rabbit which developed extensive skin lesions after intracutaneous injection of virus prepared in rabbit kidney cells (A. B. Sabin, *personal communication*). Approximately 5 ml of the serum was inactivated at 50 C for 30 min, and was absorbed three times with a lysate obtained from 1 cc each of packed uninfected HEp-2 and DK cells and once with 0.5 cc of packed sheep red blood cells. The absorptions were done with constant shaking at 37 C. Each absorption lasted 1 hr. The serum was then centrifuged at 30,000 rev/min for 2.5 hr. The supernatant fluid was collected and stored at −10 C. The antiserum reacted at a dilution of 1:160 with infected cell lysate (defined below). The serum was used at a 1:40 dilution. At this dilution, it reacted with antigens remaining in the supernatant fluid after centrifugation at 30,000 rev/min for 2 hr in a Spinco 30 rotor and with pelleted, washed virus (A. B. Sabin, *personal communication*). The serum did not react with uninfected cell lysates.

Virus. The pertinent properties of the macroplaque (MPdk$^-$) strain of herpes simplex virus have been described elsewhere (10–12). The virus was assayed in HEp-2 cells under a liquid overlay (11), and was scored in terms of PoFU.

Preparation of infected cell lysates. Monolayer cultures of HEp-2 or DK cells were exposed to virus in PBS-A-G for 2 hr at 37 C. The inoculum was then aspirated and replaced with minimal essential medium of Eagle (4) containing 1% calf serum and 13 μc/ml of thymidine-^{3}H. After 20 hr of incubation, the cells were scraped, washed with PBS-A-G, and resuspended in distilled water. After swelling for 1 hr at room temperature, the cells were lysed by 20 strokes with a tight Dounce homogenizer. Cell debris was removed by centrifugation at 1,500 rev/min for 30 min. Lysates of uninfected cells were prepared from replicate cultures overlaid for 20 hr with the same medium as the infected cells.

The Dounce homogenizer selected for this work was found to break 2% of infected cell nuclei after two strokes with the tight pestle. After 20 strokes, 50 to 70% of the nuclei were broken. Infected cell nuclei were enlarged and very fragile; microscopic examination of material subjected to 20 strokes failed to reveal whole nuclei. It should be noted that Dounce homogenization of swollen cells was used in place of freezing and thawing (11) to extract virus from infected cells for two reasons: (i) the two procedures are almost equally efficient in releasing virus from cells; (ii) we were concerned that repeated cycles of freezing and thawing would damage the structural integrity of the virus and make it more readily disassembled on isopycnic centrifugation in CsCl gradients.

Formaldehyde fixation. The cell lysates were diluted with 5× TEA buffer in the ratio of 4:1. The 28% formaldehyde solution in TEA buffer was then added to yield the desired concentration. After 16 hr at 4 to 6 C, the formaldehyde-treated cell lysates were dialyzed against TEA in the cold. Control preparations were treated in the same manner, except that TEA buffer was added instead of formaldehyde.

Isopycnic centrifugation in CsCl solution. All centrifugations were done in SW39 rotors at 15 C, 35,000 rev/min for 44 hr. The density range, approximately 1.20 to 1.60 g/cm^3, was spanned by using three tubes with overlapping density gradients. This was done by splitting the cell extracts into three portions and by adding to each portion an appropriate amount of dry CsCl. At the end of the centrifugation, the bottom of the tube was pierced and 5 two-drop fractions were collected on the prism of a Zeiss refractometer and 18 five-drop fractions were collected into SCS. The density of the CsCl was determined by reading the refractive index of the two-drop fractions. Samples of 100 μliters each were withdrawn from the 5-drop fractions collected in 1 ml of SCS and precipitated with 7.5% trichloroacetic acid on membrane filter discs (type HA; Millipore Filter Corp. Bedford, Mass.). These were then washed with 5% trichloroacetic acid, 75% ethyl alcohol containing 2% potassium acetate, and 75% ethyl alcohol; they were then oven-dried, and immersed in toluene base scintillation fluid. Tritium disintegrations were counted in a Packard scintillation spectrometer. A portion of the fraction collected in SCS was dialyzed against Veronal buffer for assay of antigenic mass by complement-fixation.

In several experiments, the identity of labeled DNA,

whether viral or cellular, was determined by measuring its buoyant density in CsCl. The DNA was extracted from pools of appropriate fractions collected in SCS with sodium lauryl sulfate by the procedure described previously (13), and then centrifuged in a CsCl solution with an initial density of 1.730 gm/cm^3 for 40 hr at 35,000 rev/min and 20 C. As shown previously (13), the buoyant density of MPdk$^-$ DNA is 1.725. The buoyant density of HEp-2 and of DK cell DNA is approximately 1.708. Preliminary experiments have shown that there is no appreciable change in the buoyant density of DNA treated with formaldehyde after extraction.

Complement-fixation tests. The general technique described by Osler et al. (8) was used. To conserve material, the tests were set up in microtiter plates (Cooke Engineering Co., Alexandria, Va.) with 0.025 ml of antigen diluted twofold in Veronal buffer, 0.025 ml of fresh guinea pig serum containing 5 C'H$_{50}$ units of complement titered in the presence of antiserum, and 0.05 ml of optimally sensitized sheep red blood cells. Anticomplementary effects of each dilution of antigen or of antiserum were tested against 3 and 5 C'H$_{50}$ units of complement. Results were recorded in terms of the degree of lysis (0, 1, 2, 3, 4, corresponding to 0, 25, 50, 75, and 100% lysis, respectively). Antigen or antiserum titers were expressed in terms of the reciprocal of the initial dilution giving 50% lysis.

RESULTS

Instability of herpes virions in CsCl. Two series of experiments illustrate the instability of the MP*dk*$^-$ herpes virion in CsCl solution. The objective of the first series was to determine the amount of infectious virus, both untreated and stabilized with formaldehyde, recovered after centrifugation in CsCl density gradients. An infected cell lysate was split into six equal portions and treated with 0, 0.55, 1.08, 3.10, 5.60, and 9.3% formaldehyde, and then was dialyzed as described in Materials and Methods. After dialysis, 100-μliter portions of treated and untreated materials were withdrawn for assay (input virus). The remainder of each portion was equalized with respect to volume and was centrifuged in CsCl for 40 hr. The initial density of the CsCl solution was 1.29 g/cm^3, i.e., higher than the buoyant density of the virus. At the end of the centrifugation, the contents of the tubes were thoroughly mixed, and then were assayed together with portions of the input materials. The recovery of infectious virus after centrifugation is shown in Table 1. The datum pertinent to this section is that approximately 85% of the infectivity of the input virus is lost on centrifugation in CsCl. The loss observed on centrifugation in CsCl is real; in the course of these studies, it has been ascertained that it cannot be accounted for by the method of storage of the input virus sample during centrifugation.

TABLE 1. *Recovery of infectious virus from untreated and formaldehyde-treated infected cell lysates after centrifugation in CsCl density gradients*

Per cent HCHO (a)	PoFU of virus recovered after formaldehyde treatment (b)	PoFU of virus recovered after centrifugation (c)	Per cent recovery after centrifugation (100 c/b)
0.00	9.5×10^6	1.4×10^6	15
0.55	11.5×10^3	14.6×10^3	>100
1.08	0.9×10^3	14.9×10^3	>100
3.10	6.8×10^3	16.9×10^3	>100
5.60	2.7×10^3	9.5×10^3	>100
9.30	3.7×10^3	11.3×10^3	>100

The second series of experiments demonstrated the instability of herpes virions in CsCl solution by showing that the total recovery of labeled virions is considerably improved by pretreatment with formaldehyde. In this series of experiments, lysates of infected cells labeled with thymidine-^{3}H were treated with various concentrations of formaldehyde, and then were centrifuged in a CsCl density gradient. The recovery of thymidine-^{3}H label in the various fractions is shown in Fig. 1. The datum pertinent to this section is that the recovery of labeled virus can be increased fivefold by optimal pretreatment with formaldehyde. Two additional findings should be noted. First, the pellet at the bottom of the various tubes contained labeled cellular and viral DNA.

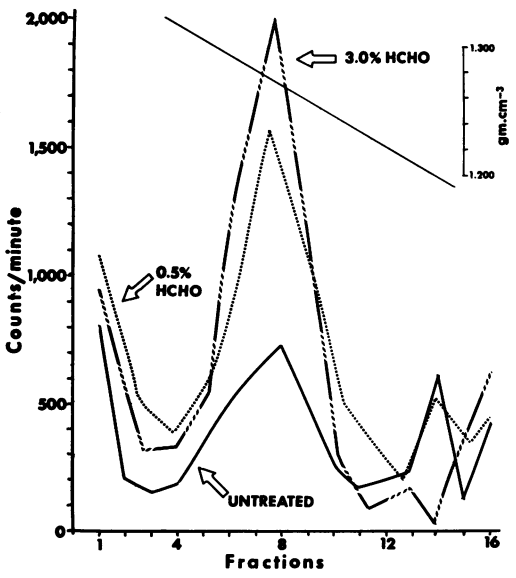

FIG. 1. *Effect of pretreatment of lysates of infected HEp-2 cells with formaldehyde on the recovery and distribution of herpes virions labeled with thymidine-^{3}H in CsCl gradients.*

The pellets obtained after centrifugation of formalinized material contained one-third to one-half as much radioactivity as pellets obtained after centrifugation of untreated material. The second finding concerns the identity of DNA banding at the density of the virus. The DNA was extracted with sodium lauryl sulfate (13) from material originally banding between the 1.25 and 1.30 g/cm³ bands in CsCl at a density characteristic of viral DNA; host DNA does not band in the 1.25 to 1.30 density range.

It should be pointed out that there is good agreement between the loss of infectivity on centrifugation on one hand and the improvement of recovery of labeled virus from gradients by pretreatment with formaldehyde on the other. This finding suggests that the inactivation of infectivity may be due to disassembly of the virion into its structural constituents. The data suggest, moreover, that the bulk of the virions are stabilized by formaldehyde in CsCl gradients.

Characteristics of formaldehyde treatment and properties of stabilized virions. The data concerning the properties of the stabilized herpes virions emerged from three series of experiments. The first, summarized in Table 1, shows the following: (i) the infectivity of the virus was markedly reduced by treatment with formaldehyde; (ii) the inactivation was probably not a pseudo first-order reaction as evident from the fact that the amount of residual infectious virus was not affected greatly by changes in formaldehyde concentration from 0.55 to 9.3%; (iii) the residual infectious virus in formaldehyde-treated preparations increased in titer after ultracentrifugation in CsCl. Again, the extent of increase was not related to the concentration of formaldehyde to which the virus was exposed.

The second series of experiments concerned the buoyant density of stabilized virus. Generally, the bulk of the untreated infectious virus bands in the density range of 1.26 to 1.27 g/cm³. In a number of experiments, the buoyant density of stabilized virus was found to be higher: between 1.280 and 1.285 g/cm³.

The third series of experiments concerned the optimal conditions for viral stabilization. The procedure for ribosome stabilization by formaldehyde (9) recommended buffering the reaction mixture at *p*H 7.75. In this series of experiments, portions of a lysate of infected HEp-2 cells labeled with thymidine-³H were exposed to different concentrations of formaldehyde at *p*H 7.05 or at 7.75. After treatment, the lysates were dialyzed against TEA buffered at the corresponding *p*H, and then were centrifuged in CsCl solutions. The fractions collected after centrifugation were assayed for radioactivity. Table 2 shows the recovery of ³H-labeled material in the virus band (1.25 to 1.30 g/cm³). The data presented in Table 2 show that optimal recovery of virus may be achieved with 3.1% formaldehyde buffered at *p*H 7.05. These conditions were used to stabilize virus in cell lysates in all subsequent experiments.

TABLE 2. *Effect of concentration and pH of formaldehyde on the recovery of thymidine-³H labeled virus on isopycnic centrifugation in CsCl*

Per cent HCHO	Counts/min of thymidine-³H labeled virus precipitated by trichloroacetic acid			
	Expt 1		Expt 2	Expt 3
	*p*H 7.05	*p*H 7.75	*p*H 7.05	*p*H 7.75
0.00	3,100	3,200	600	2,750
0.55	ND[a]	ND	ND	4,750
1.08	ND	ND	3,300	4,950
3.10	15,600	13,050	3,300	5,200
5.60	12,700	13,500	2,800	5,800
9.30	ND	ND	2,800	4,600
14.00	ND	ND	2,100	ND
Ratio of trichloroacetic acid-precipitable counts: 3.1% HCHO-treated/untreated	5.0	4.1	5.3	1.9

[a] Not done.

Analysis of the products of productive and abortive infection by isopycnic centrifugation. Lysates from approximately 4×10^8 infected HEp-2 cells and from an equal number of labeled uninfected cells were each split in half. One half was treated with formaldehyde, and then centrifuged in three CsCl density gradients spanning the range from 1.2 to 1.6 g/cm³. The other half of each lysate was centrifuged untreated. The experiments were repeated with lysates of DK cells. The viral or cellular identity of the DNA in material banding at different densities was determined after extraction by measuring its buoyant density as described in Materials and Methods. To facilitate the presentation of the results of various assays, the distributions of antigen and of thymidine-³H labeled DNA in the three gradients were combined in a single figure each for HEp-2 cells (Fig. 2) and DK cells (Fig. 3). Materials in the top fractions and pellets are now shown. The results were as follows.

(i) Nonformalinized material containing DNA from uninfected cell lysate did not band in the density range of 1.2 to 1.6 g/cm³ (Fig. 2A, 3A).

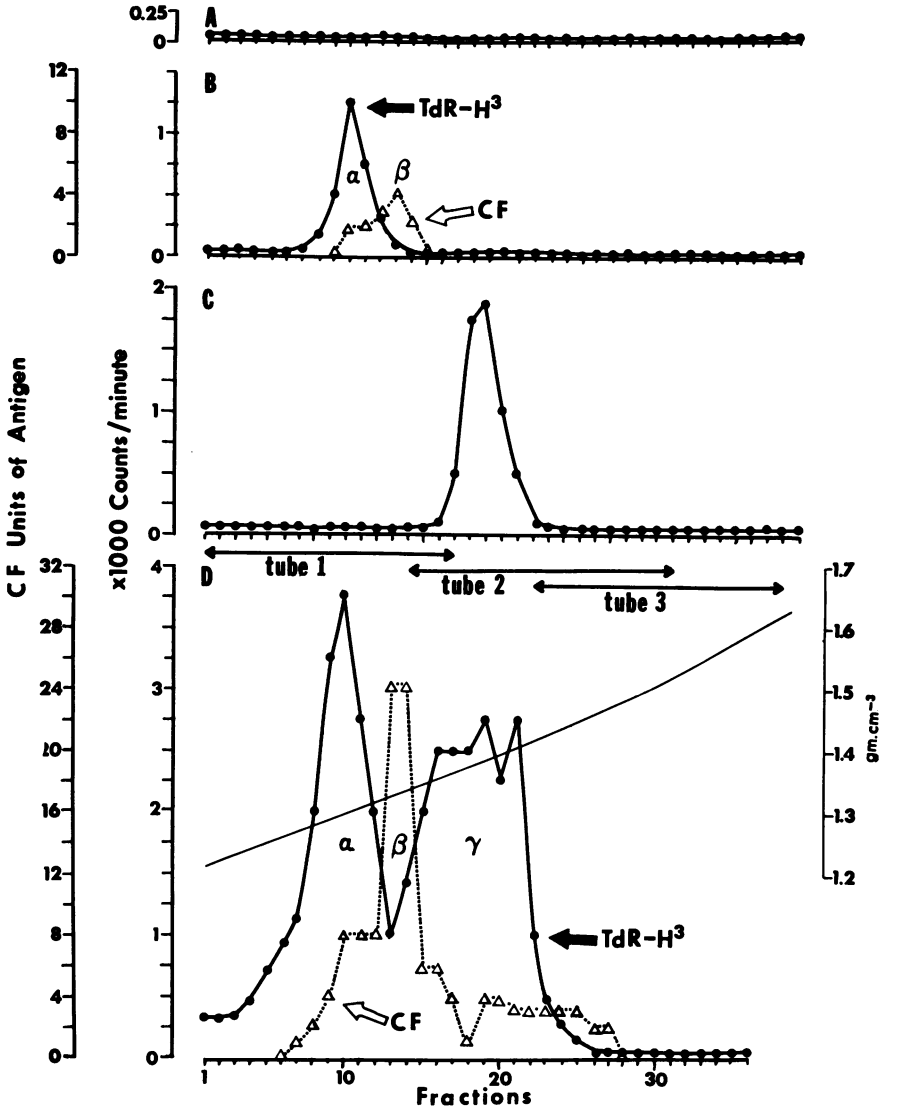

FIG. 2. *Distribution of radioactive label and complement-fixing antigen on isopycnic centrifugation in CsCl solutions of lysates of HEp-2 cells labeled with thymidine-³H. (A) Untreated lysates of uninfected cells; (B) untreated lysate of infected cells; (C) formalinized lysates of uninfected cells; (D) formalinized lysates of infected cells. Each distribution is reconstructed from data obtained from three overlapping gradients. The density range covered by each gradient is shown by arrows labeled tube 1, tube 2, and tube 3.*

(ii) Formalinized material from uninfected cell lysates containing cellular DNA did band in the density range spanned by the gradients. Material containing HEp-2 cell DNA banded in one position at an average density of 1.390 g/cm³ (Fig. 2C). Formalinized material containing DK cell DNA formed a major band with an average density of 1.410 g/cm³ and several minor bands with densities of 1.30 and 1.33 g/cm³ (Fig. 3C).

(iii) Nonformalinized extracts of infected HEp-2 cells yielded (Fig. 2B) a band, designated α, which contained infectious virus, viral DNA, and complement-fixing antigen and a band, designated β, which contained predominantly complement-fixing antigen and little thymidine-³H labeled DNA. The average densities of α and β bands were 1.270 and 1.305 g/cm³, respectively. Nonformalinized extracts of infected DK cells (Fig. 3B) did not yield viral material banding

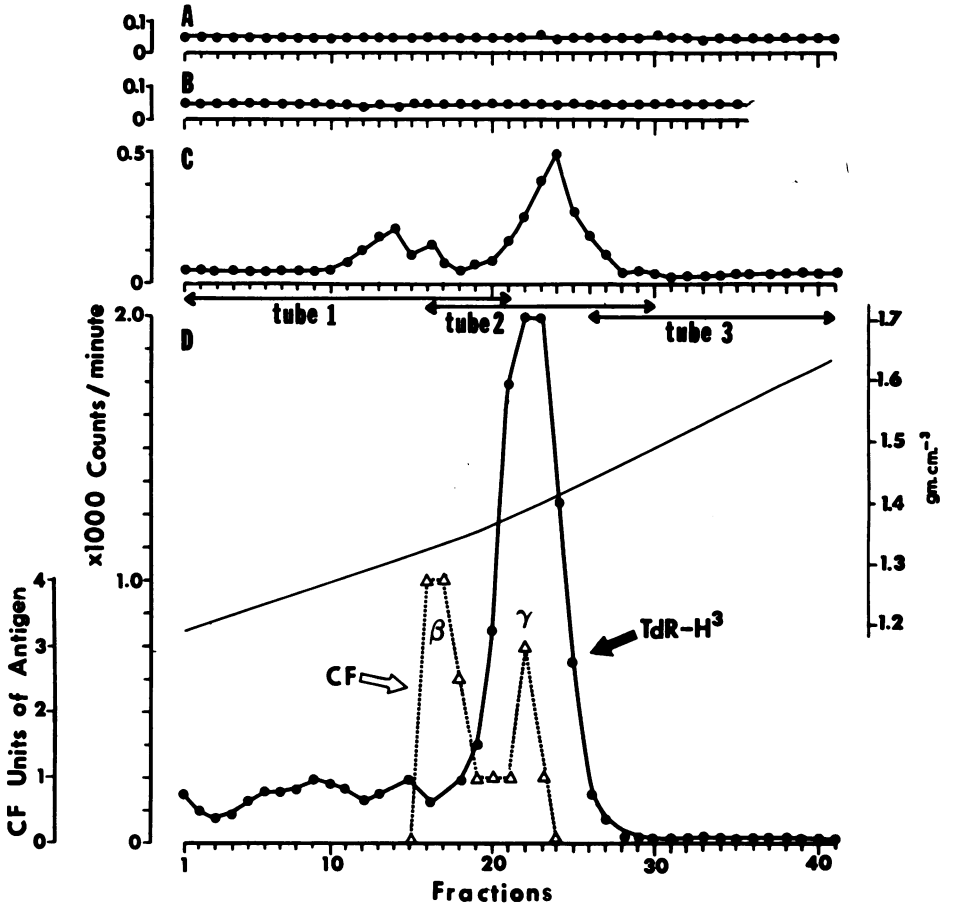

FIG. 3. *Distribution of radioactive label and complement-fixing antigen on isopycnic centrifugation in CsCl solutions of lysates of DK cells labeled with thymidine-³H. (A) Untreated lysate of uninfected cells; (B) untreated lysate of infected cells; (C) formalinized lysate of uninfected cells; (D) formalinized lysate of infected cells. Each distribution is reconstructed from data obtained from three overlapping gradients. The density range covered by each gradient is shown by arrows labeled tube 1, tube 2, and tube 3.*

at the densities corresponding to those of α and β bands.

(iv) Formalinized lysates of infected HEp-2 cells (Fig. 2D) yielded three bands: α, with a mean density of 1.285 g/cm³, containing viral DNA and complement-fixing antigen; β, with a mean density of 1.325 to 1.330 g/cm³, containing primarily complement-fixing antigen; and a very broad band, designated as γ, with a density range of 1.37 to 1.45. The γ band was shown to contain complement-fixing antigen and both viral and cellular DNA. Formalinized lysates from infected DK cells (Fig. 3D) yielded fewer bands with somewhat different properties. Thus, the α band was completely missing. The β band had a mean density of 1.325 g/cm³ and was similar to that obtained from infected HEp-2 cell extracts. The γ band was characteristically narrower; it had a buoyant density of 1.39 g/cm³, and, moreover, the position of complement fixing antigen and of thymidine-³H labeled DNA corresponded much better in the γ band obtained from infected DK cells than in that of HEp-2 cells.

DISCUSSION

We have presented evidence that (i) herpes simplex virions become disassembled in CsCl solution, (ii) the virions may be stabilized against breakdown by pretreatment with formaldehyde, and (iii) in abortive infection some viral products are either absent or they have different properties from corresponding products made in productively infected cells. Some implications and

consequences of our results can be discussed as follows.

Instability of herpes simplex virus in CsCl solutions. The basic finding of this study is that herpes simplex virus becomes disassembled in CsCl solutions. This conclusion is based on the finding that the amount of CF antigen and labeled DNA banding at the density of virus increases on pretreatment with formaldehyde. We are not certain whether the inactivation is due to CsCl alone or to the combined effects of CsCl and physical stress exerted on the virion during centrifugation. The implications are several, and the conclusions affect all studies relating biological activity and sedimentation behavior, in which quantitative recovery of herpes virions is paramount. We are referring in particular to the study by Smith (15) purporting to show that infectivity is a function of enveloped particles only. The conclusion is based on the finding that infectivity correlated with the presence of enveloped virions in fractions obtained after isopycnic centrifugation in CsCl. Smith's study makes the implicit assumption that centrifugation in CsCl solutions is not deleterious, or, at least, that CsCl does not selectively degrade one form of the virus in preference to the other. Our data show that the virion is degraded in CsCl solutions. We must conclude, therefore, that at the moment there is no basis for differentiating enveloped and unenveloped virus with respect to biological activity.

Stabilization of herpes virions by formaldehyde. The subunits of the nucleocapsid are probably held together by electrostatic rather than covalent bonds. These bonds are usually disrupted by the high ionic strength of the gradient. Formaldehyde reacts with the amino groups of purine and pyrimidine bases (14) and with the amino groups, imino groups, ring structures, sulfhydryl groups, and hydroxyl groups of proteins (5, 6). It is not clear which of these reactions is primarily responsible for the loss of infectivity. However, it seems most likely that formaldehyde stabilized the virion by cross-linking adjacent nucleocapsid subunits by methylene and polymethylene bridges (5, 6).

Properties of stabilized products of productively infected cells. The formalinized herpes virion (α band) has a mean buoyant density approximately 0.015 g/cm^3 higher than that of the nonformalinized virion. Several hypotheses could account for this shift: (i) the formalinized virion is less permeable and reacts differently with the CsCl, (ii) formaldehyde stabilizes a large population of virions characterized by a relatively higher density and increased lability in CsCl tions. We cannot differentiate between these hypotheses, but it is of interest to note that the material in the β band also increases in buoyant density by approximately the same amount following formaldehyde treatment.

We have no information concerning the nature of the material in the β band, other than the fact that it appears to contain relatively little if any DNA. The material in the γ band is of interest from several points of view: (i) it is detected only on centrifugation of formalinized lysates, suggesting that it is very labile; (ii) it is heterogeneous with respect to buoyant density; and (iii) it contains viral DNA, complement-fixing antigen, and host DNA. Some of the material in the γ band is undoubtedly stabilized host DNA-protein complexes. The viral components of the γ band fit, a priori, the expected behavior of partially assembled nucleocapsids. Whether this is, in fact, the case remains to be seen.

Products of abortively infected cells. The most interesting aspect of this study is the observation that MPdk$^-$ herpes virions could not be detected even in formalinized lysates of infected DK cells. We must conclude that these virions are either not made at all or that they are more labile than the DNA-antigen complex in the γ band. One obvious question that arises is whether the absence of virions is related to the anomalous sedimentation behavior of the material in the γ band. The answer to this question also remains to be seen in the electron microscope.

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