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Defective Interfering Particles of Poliovirus

IV. Mechanisms of Enrichment

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Infection of HeLa cells by mixtures of standard poliovirus and defective, interfering (DI) poliovirus particles leads to a higher ratio of DI particles in the progeny than in the inoculum. The extent of this enrichment could be varied by various manipulations of the co-infected cells. At any time during the infection cycle, virions made within short times after addition of radioactive uridine were hyperenriched in DI particles; this transient hyperenrichment fell to the equilibrium enrichment level within 45 min after uridine addition. A shift of the temperature of infection from 37 to 31 C also led to a hyperenrichment of DI particles and pulse-labeling revealed a superimposed transient hyperenrichment. By contrast, cells continuously infected at 31 C showed a severe decrement in DI particles apparently because poliovirus DI particles behave as cold-sensitive mutants for RNA synthesis. Cycloheximide treatment early in the infection cycle also led to hyperenrichment. Study of the cycloheximide effect showed that the drug acted as if to change the input ratio of standard to DI particles. These effects on enrichment can be explained as aspects of two different phenomena: enrichment due to preferential DI RNA synthesis and enrichment due to preferential encapsidation of DI RNA. Both mechanisms probably play a role in the normal level of enrichment.

The isolation and characterization of poliovirus DI particles was reported previously (4). These particles lack 13 to 17% of the poliovirus genome, are less dense in CsCl gradients than standard poliovirus, and can be obtained less than 1% contaminated by standard virus using CsCl gradients (2, 4). Poliovirus DI particles appear to carry a deletion in that part of the genome specifying virion capsid protein and therefore require the presence of standard poliovirus for their growth (2). Cells infected by both standard poliovirus and DI particles produce less standard poliovirus than is produced when cells are infected only by standard poliovirus. This interference appears to result from a competition between DI and standard particles for the limited biosynthetic capacities of the infected cell (3).

Not only do DI particles interfere with the growth of standard virus but also the progeny yield from co-infected cells is always enriched about 5 to 8% in DI particles compared to the infecting virus mixture (3). This enrichment is the most important biological property of DI particles because it allows DI particles to predominate over standard virus after multiple passages. Unfortunately, enrichment has been impossible to study directly because DI RNA and standard RNA are not sufficiently separable by known methods to allow precise quantitation of their proportion in infected cells. Therefore, indirect experiments must be used to study enrichment.

The following experiments were directed at examining the conditions under which enrichment occurs or is altered and the possible mechanisms of enrichment. Our results indicate that two mechanisms of enrichment occur: enrichment due to preferential replication of the shorter DI RNA and enrichment due to preferential encapsidation of DI RNA. Experiments in which virus is harvested very soon after pulse-labeling and experiments utilizing cultures shifted from 37 to 31 C suggest that preferential encapsidation can be a factor in the enrichment process. Experiments utilizing cycloheximide suggest the involvement of preferential replication in the enrichment process. The enrichment observed in normal 37 C cultures is probably a result of both effects.

MATERIALS AND METHODS

All materials and methods have been described in detail elsewhere (2, 4) and will only be outlined here.

Suspension cultures of HeLa cells and Mohoney poliovirus type 1 were used in all experiments (1). The initial isolate of poliovirus DI particles, called DI(1) (4), was used throughout except where specifically noted. Preparations of DI(1) particles containing less than 1% of standard poliovirus were prepared by CsCl centrifugation as described (2). Adsorption of virus to cells was carried out by addition of appropriate amounts of standard virus and DI particles to cells concentrated to 8×10^7 per ml in medium lacking serum followed by incubation at 22 C for 30 min. Cells were then diluted to 4×10^6 per ml with medium plus 5% horse serum (defined as time 0). Protocols for addition of inhibitors and radioactive compounds are indicated for the individual experiments. For analysis of progeny virions, infected cells were harvested, disrupted by freezing and thawing and sodium dodecyl sulfate was added to 0.5%. The radioactive virions were collected by ultracentrifugation, resuspended, and analyzed by centrifugation to equilibrium in CsCl gradients (4). Fractions of the gradient were precipitated with 10% trichloroacetic acid, the acid-insoluble material was collected on Millipore filters and radioactivity was determined by scintillation counting.

RESULTS

Transient hyperenrichment. Previously reported experiments demonstrated that progeny virus from cells infected by standard virus and DI particles is produced in the same ratio throughout the period of virus production. In those experiments, radioactive uridine was added to co-infected cultures at various times during the infection cycle and virus was harvested 50 min later (3). In order to study the labeled virus formed just after addition of radioactive uridine, infected cells were exposed to ^{3}H-uridine at 150 min after infection and progeny virions were harvested from 8 to 45 min after the addition of label. To analyze the progeny, the particles were mixed with virions which had been labeled with ^{14}C-uridine throughout the virus growth cycle and the mixtures were sedimented to equilibrium in CsCl density gradients. It can be seen that virions produced after 8 min of exposure to ^{3}H-uridine (Fig. 1A) contained a much greater proportion of DI particles than the continuously labeled progeny. As the length of the labeling period was increased, the proportion of DI particles in the accumulated progeny decreased and the extent of enrichment of DI particles in the progeny approached an equilibrium level (Fig. 1B–1E). This equilibrium enrichment is the amount of enrichment observed in the progeny yield from the whole infection cycle as well as in the virions made during any 50-min period after 120 min of infection (3). Pooled data from a number of experiments showed that a 45-min exposure to label is necessary to attain the equilibrium level (Fig. 2). Thus, there is transient hyperenrichment for DI particles under pulse-labeling conditions. Virus produced after exposure to ^{3}H-uridine for increasing periods of time during three different periods of the infection cycle, 120 to 165, 165 to 210, and 180 to 235 min after infection, displayed approximately the same transient hyperenrichment and kinetics of approach to equilibrium as shown in Fig. 2.

These experiments were also performed using DI(3) particles (4), an independent isolate of poliovirus type 1 DI particles which behaves very similarly to DI(1) particles. DI(3) particles were also enriched in the progeny yield from a mixed infection approximately 5 to 8% relative to their proportion in the infecting virus mixture and they displayed transient hyperenrichment very similar to that seen with DI(1) particles (data not shown).

Hyperenrichment by temperature shift. A greater extent of enrichment than the normal equilibrium enrichment was also observed when co-infected cells were shifted to temperatures lower than 37 C at 150 min after infection. To demonstrate this effect, cells were infected by a mixture of standard and DI particles and incubated at 37 C. After 150 min, portions of this culture were shifted to 31, 34, and 39 C, or kept at 37 C, and immediately exposed to ^{3}H-uridine. A separate portion of the culture maintained at 37 C was exposed to ^{14}C-uridine. Five hours later, virus was harvested and the ^{3}H-uridine-labeled progeny were mixed with ^{14}C-uridine-labeled progeny and sedimented to equilibrium in CsCl density gradients (Fig. 3). A large increment in DI particles was produced after a shift to 31 C, a smaller hyperenrichment was produced after a shift to 34 C, and virus produced after a shift to 39 C contained approximately the same or a slightly lower proportion of DI particles as the equilibrium enrichment mixture produced by constant incubation at 37 C.

In order to determine whether transient hyperenrichment could be superimposed on hyperenrichment caused by a temperature shift, the following experiment was performed. A culture of cells was transferred from 37 C to 31 C at 2.5 h after infection. After the shift, one-half was exposed to ^{14}C-uridine for 5 h and the other half was exposed to ^{3}H-uridine for shorter periods of

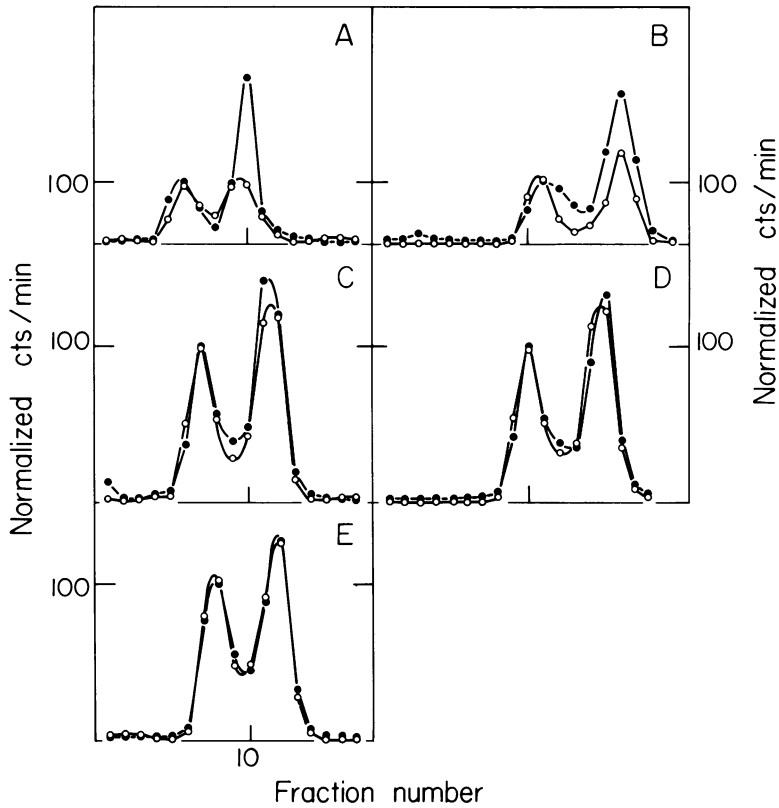

FIG. 1. *CsCl density gradients of the progeny of standard and DI particle co-infected cells labeled for various times with ^{3}H-uridine. A culture of 1.6 × 10^8 HeLa cells in 40 ml was infected with a mixed stock of standard and DI(1) particles in the presence of actinomycin D (5 μg/ml). At 60 min after infection, one-half of the cultures was labeled with ^{14}C-uridine (20 μCi) and labeling continued for 5 h. At 165 min after infection, 4 ml of medium containing 2 mCi of ^{3}H-uridine was added to the other half of the culture. Portions of this culture were removed at various times, washed once with Earle saline, and resuspended in 1 ml of Earle saline. Virus was released by three cycles of freezing and thawing. A portion of the ^{14}C-uridine-labeled culture was added to each ^{3}H-uridine-labeled sample and virus examined in CsCl gradients, as described (4). The peak of standard virus (ρ = 1.34 g/cc) is at the left in this and all other figures and the DI particle peak (ρ = 1.325 gm/cc) is at the right (4). In each case the data were normalized by setting the peak of standard virus equal to 100 for both ^{14}C- and ^{3}H-uridine label. The duration of labeling was (A) 7 min; (B) 16 min; (C) 24 min; (D) 36 min; and (E) 45 min. Symbols: ○, ^{14}C-uridine-labeled virus; ●, ^{3}H-uridine-labeled virus.*

time (Fig. 4). ^{3}H- and ^{14}C-uridine-labeled progeny were mixed and sedimented to equilibrium in CsCl density gradients. As seen before in cultures maintained at 37 C, virus harvested 12 min after the addition of ^{3}H-uridine (Fig. 4A) was hyperenriched and equilibrium was approached as the labeling period was extended to 28 min (Fig. 4B), 45 min (Fig. 4C), or 60 min (Fig. 4D). Thus, transient hyperenrichment can occur along with temperature shift-mediated hyperenrichment. Since the overall rate of progeny virus formation is lower at 31 C than at 37 C (Cole, unpublished results), the attainment of equilibrium requires 60 min at 31 C rather than the 45 min required at 37 C.

Decrement in DI particles due to infection at temperatures other than 37 C. In contrast to the hyperenrichment observed after temperature shifts, incubation of co-infected cells continuously at temperatures other than 37 C resulted in reduced proportions of DI particles in the progeny (Fig. 5 and Table 1). Incubation at 31 C yielded progeny containing only 15% DI particles. At 34 C there was less decrement in the DI particle yield, with the progeny containing 32% DI particles compared to the 43% obtained at 37 C. Only a slight decrement (to 39%) was seen at 39 C.

In order to examine whether the decreased DI particle formation at 31 C resulted from a

decreased amount of DI RNA synthesis, we have studied the incorporation of ^{14}C-uridine into infected cultures of cells at different temperatures. DI particle-infected cells incorporated only 15% as much uridine as standard virus-infected cells at 31 C (Fig. 6), whereas both standard and DI particle-infected cultures incorporated identical amounts of uridine at 37 C (2). If cultures were shifted from 37 to 31 C at 2 h after infection, both standard and DI particle-infected cultures continued to incorporate ^{14}C-uridine into viral RNA to approximately the same extent (Fig. 6). Thus, DI particles are both deletion mutants in the capsid protein cistron and cold-sensitive mutants for early RNA synthesis. The cold-sensitivity is apparently not manifested after the first 2 h of infection.

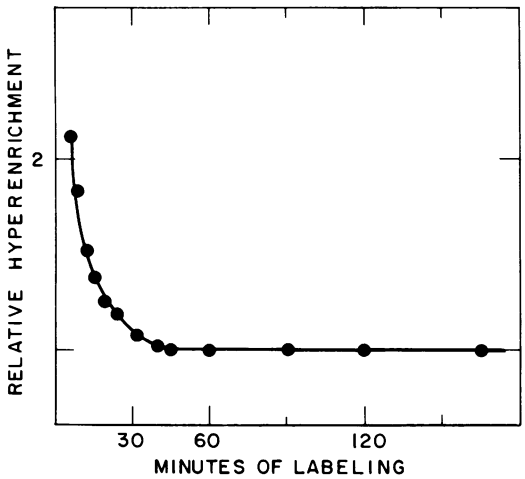

FIG. 2. *Relative hyperenrichment in DI particles. Relative hyperenrichment at different times was determined from the normalized data in Fig. 1 and other similar experiments. Relative hyperenrichment is the ratio of the integrated area of the DI particle peak at any time divided by the integrated area of the DI particle peak at equilibrium.*

Effect of cycloheximide treatment on enrichment. The following experiments were performed to ascertain whether inhibition of protein synthesis during the early stage of the infection cycle could change the extent of enrichment. The concept behind these experiments, which is developed more fully in the Discussion, was that enrichment might be partially determined during the early period of the infection when the amount of viral RNA polymerase limits the rate of RNA synthesis. Inhibition of protein synthesis at an early time in the

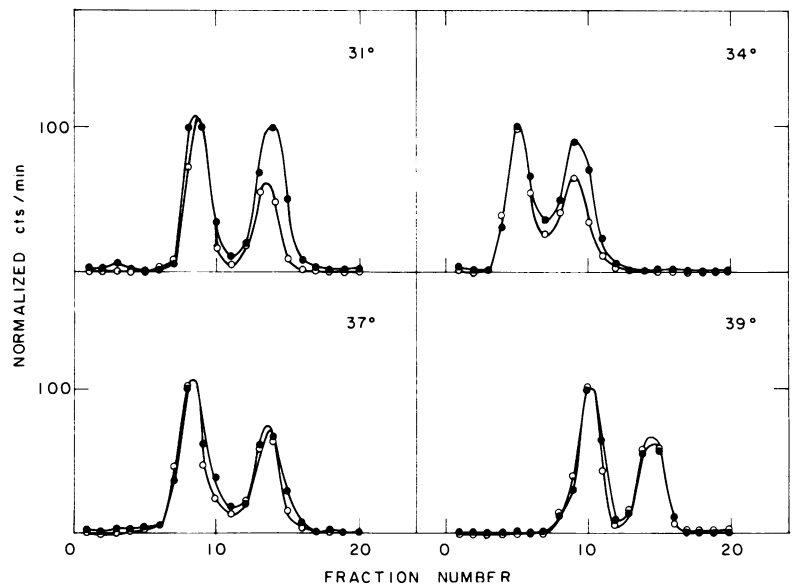

FIG. 3. *CsCl density gradients of the progeny from standard and DI particle co-infected cells shifted to different temperatures at 150 min after infection. A culture of 2.4 × 10^8 cells in 60 ml was infected with a mixed stock of standard and DI(1) particles in the presence of actinomycin D (5 μg/ml). At 60 min after infection, one-third of the culture was labeled with ^{14}C-uridine (20 μCi) and labeling continued for 300 min. At 150 min after infection 10-ml portions of the culture were transferred to 31, 34, or 39 C or maintained at 37 C and labeled with 0.25 mCi of ^{3}H-uridine. Virus was harvested 300 min later by three cycles of freezing and thawing. A portion of the ^{14}C-uridine-labeled culture was added to each ^{3}H-uridine-labeled culture. The virus was examined in CsCl gradients and the data was normalized as described in the legend to Fig. 1. Symbols: ○, ^{14}C-uridine-labeled virus; ●, ^{3}H-uridine-labeled virus.*

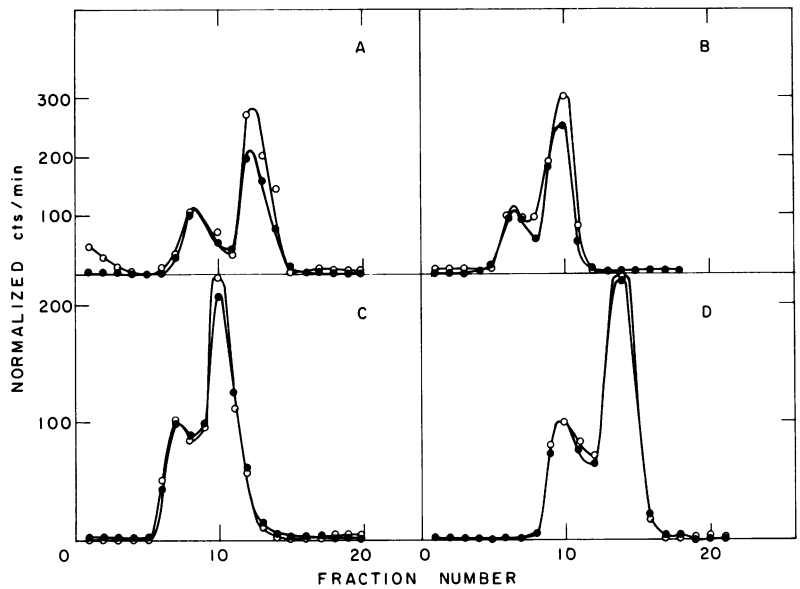

FIG. 4. *CsCl density gradients of the progeny of standard and DI(1) particle co-infected cells showing both transient and temperature-dependent hyperenrichment. A culture of 8 × 10⁷ cells in 20 ml, infected with a mixed stock of standard and DI(1) particles in the presence of actinomycin D (5 μg/ml), was incubated at 37 C. At 150 min after infection, the culture was transferred to 31 C and half of the culture was exposed to ¹⁴C-uridine (10 μCi) for 300 min. The other half received ³H-uridine (1 mCi in 2 ml of medium). Portions of this culture were removed at various times, washed once with Earle saline, and resuspended in 1 ml of Earle saline. Virus was released by three cycles of freezing and thawing. A portion of the ¹⁴C-uridine-labeled culture was added to each ³H-uridine-labeled sample and virus examined in CsCl gradients. The data was normalized as described in the legend to Fig. 1. Symbols: ●, ¹⁴C-uridine-labeled virus; ○, ³H-uridine-labeled virus. The duration of labeling was (A) 12 min; (B) 28 min; (C) 45 min; and (D) 60 min.*

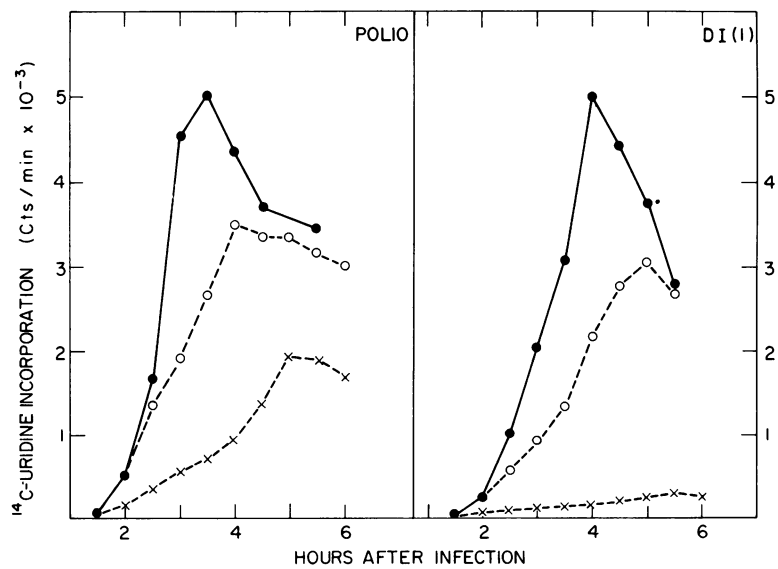

FIG. 5. *Effect of temperature of incubation on the incorporation of ¹⁴C-uridine into viral RNA in cultures of cells infected by either standard or DI(1) particles. Two cultures of 3 × 10⁷ cells in 7.5 ml were infected with either standard or DI(1) particles in the presence of actinomycin D (5 μg/ml). The multiplicities of infection were 20 plaque-forming units per cells for the standard virus-infected culture, and 12.5 plaque-forming unit equivalents (2) per cell for the DI(1) particle-infected culture. One-third of each culture was incubated at 31 C, one-third was incubated at 37 C, and one-third was incubated at 37 C for 120 min and then shifted to 31 C. ¹⁴C-uridine (0.1 μCi/ml) was added to each culture 60 min after infection. Every 30 min, beginning 90 min after infection, 0.1-ml portions were withdrawn from each portion and acid-precipitable radioactivity was determined. The data has been corrected for the incorporation of ¹⁴C-uridine into an actinomycin D-treated, mock-infected culture. Symbols: ×, 31 C cultures; ●, 37 C cultures; ○, cultures shifted from 37 to 31 C 120 min after infection.*

infection cycle might then elongate the period during which polymerase is limiting. Co-infected cells were therefore incubated at 37 C in the presence of 100 μg of cycloheximide per ml, a potent inhibitor of mammalian protein synthesis (5, 8), for different 60-min intervals during the infection cycle. After removal of the drug, ^{3}H-uridine was added to each culture and the labeled progeny was purified in CsCl gradients, along with ^{14}C-uridine-labeled progeny produced in a parallel culture which did not receive the drug. Representative data from the experiment are shown in Fig. 7 and the percentage of the progeny as standard or DI particles in all of the samples is given in Table 2. Treatment with cycloheximide acted to increase the proportion of DI particles regardless of the portion of the infection cycle during which the drug was present. The effect, however, was greatest early during infection and was maximal 13 min after the initiation of infection. Treatment at this time led to progeny in which 75% were DI particles compared to 54% in the control culture which received no cycloheximide. Addition of the drug as late as 180 min after infection still led to a slight increase in the proportion of DI particles in the progeny.

TABLE 1. *Decrement in DI particles due to infection at temperatures other than 37 C*[a]

Temp of infection (C)	Distribution of progeny virus[b]	
	Standard virus (%)	DI(1) particles (%)
31	85	15
34	68	32
37	57	43
39	61	39

[a] Data were taken from Fig. 5.

[b] The amount of radioactivity in standard virus or defective particles was determined from CsCl gradients (4) and expressed as a percentage of the total labeled progeny from the culture. These values have been corrected for the smaller genome size of DI particles.

In a similar series of experiments, the effect of varying the time of cycloheximide treatment was investigated. Table 3 shows that longer exposure to cycloheximide resulted in an increased percentage of DI particles in the progeny. Even a 10-min exposure, however, led to an 8 or 9% increase in the percentage of DI particles compared to the untreated control.

The concentration of cycloheximide also af-

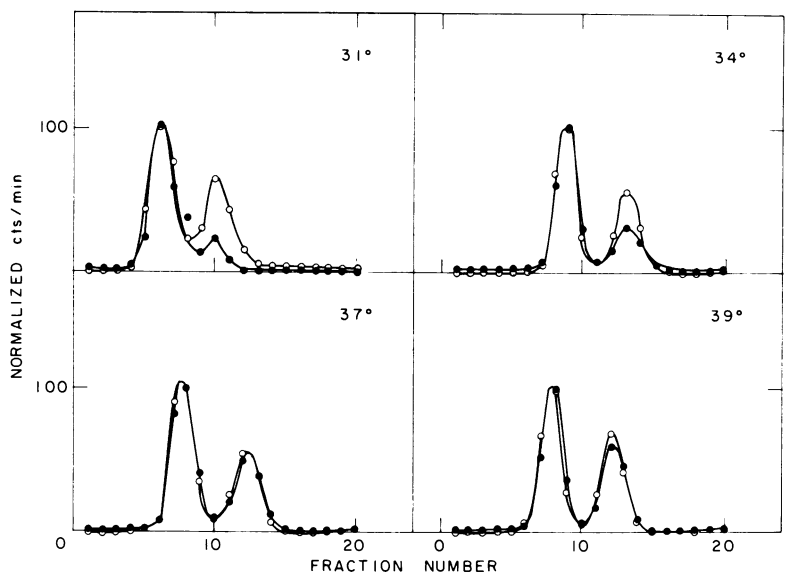

FIG. 6. *CsCl density gradients of the progeny of standard and DI particle co-infected cells incubated continuously at various temperatures. A culture of 2.4 × 10^8 cells was infected with a mixed stock of standard and DI(1) particles in the presence of actinomycin D (5 μg/ml). Portions of the culture (10 ml) were incubated at 31, 34, 37, or 39 C. Beginning 60 min after infection, they were labeled with ^{3}H-uridine (2.5 mCi/culture). The remaining 20-ml portion was incubated at 37 C and labeled with ^{14}C-uridine (20 μCi) beginning 60 min after infection. Virus was harvested after 480 min (31 C culture), 420 min (34 C culture), or 360 (37 or 39 C cultures). A portion of the ^{14}C-uridine-labeled culture was added to each ^{3}H-uridine-labeled culture and virus examined in CsCl gradients. The data was normalized as described in the legend to Fig. 1. Symbols: O, ^{14}C-uridine-labeled virus; ●, ^{3}H-uridine-labeled virus.*

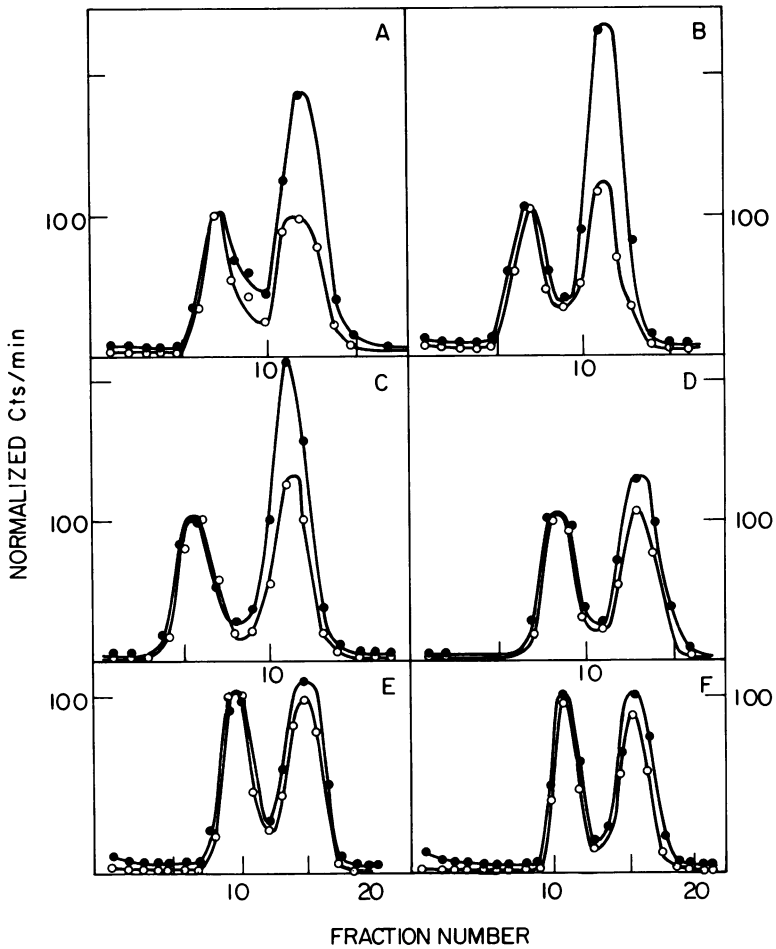

FIG. 7. *CsCl density gradients of the progeny of standard and DI(1) particle co-infected cells transiently treated with cycloheximide. A culture of 8 × 10⁸ cells in 200 ml was infected with a mixed stock of standard and DI(1) particles in the presence of actinomycin D (5 μg/ml). Eighty milliliters of this culture received ¹⁴C-uridine (80 μCi) at 60 min after infection. Portions of the culture (10 ml) were exposed to cycloheximide (Calbiochem, 100 μg/ml) for 60 min at various time after infection. Cycloheximide was removed by washing cells twice with Earle saline and cells were resuspended in normal medium plus 5% horse serum, containing ³H-uridine (0.25 mCi/culture). Virus was harvested 420 min after infection and released by three cycles of freezing and thawing. A portion of the ¹⁴C-uridine-labeled culture was added to each ³H-uridine-labeled culture, and virus was examined in CsCl gradients. The data was normalized as described in Fig. 1. The time of addition of cycloheximide was (A) 0 min; (B) 13 min; (C) 30 min; (D) 45 min; (E) 120 min; and (F) 180 min. Symbols: ○, ¹⁴C-uridine-labeled virus; ●, ³H-uridine-labeled virus.*

fected the extent of enrichment (Table 4). Cycloheximide in the amount of 10 μg/ml resulted in a small enrichment, but a much greater effect was obtained when 100 μg/ml or 200 μg/ml were used.

In order to determine whether transient hyperenrichment could be demonstrated in these cultures after reversal of cycloheximide, the same methodology used previously was employed (see Fig. 1). It can be seen (Fig. 8) that transient hyperenrichment does occur in cultures treated with cycloheximide at 20 min after infection. Similar results were obtained when cycloheximide was added at the time of infection (not shown).

Thus, treatment of co-infected cultures with cycloheximide under a variety of conditions of amount, duration of exposure, and time of addition, resulted in increased proportions of DI particles in the progeny yields. In order to attempt to understand the hyperenrichment observed after cycloheximide treatment, virus-

TABLE 2. *Hyperenrichment in DI(1) particles resulting from cycloheximide treatment at various times during the infection cycle*[a]

Time of cycloheximide addition (min after infection)	Distribution of progeny virus[b]	
	Standard virus (%)	DI(1) particles (%)
0	32	68
3	30	70
7	28	72
13	25	75
20	27	73
30	30	72
45	36	64
60	41	59
120	43	57
180	43	57
Untreated[c]	46	54

[a] From an actinomycin D treated culture of 8×10^8 cells in 200 ml, infected with a mixed stock of standard and DI(1) particles, 10-ml portions were withdrawn at the times indicated and treated with 100 μg of cycloheximide per ml for 60 min. Cycloheximide was removed by washing cells twice with Earle saline, and cells were resuspended in medium plus 5% horse serum containing ^{3}H-uridine (0.25 mCi/culture). Virus was harvested 420 min after infection and released by three cycles of freezing and thawing. A portion of the culture was exposed to ^{14}C-uridine for 6 h and samples from this culture were added to each ^{3}H-uridine-labeled culture. Virus was examined in CsCl gradients as described (4).

[b] See footnote b, Table 1.

[c] Since each gradient contained ^{14}C-uridine-labeled virus as a control, values were computed from each gradient pattern and averaged.

specific macromolecular synthesis was investigated in cells which had been exposed to the drug.

RNA and protein synthesis in cells which had been treated with cycloheximide. Cultures of co-infected cells, treated with actinomycin D, were exposed to cycloheximide (100 μg/ml) for various periods (10 to 120 min) beginning at the time of infection or 20 min after infection. A control culture received no cycloheximide. Each culture received ^{14}C-uridine and its incorporation into viral RNA was followed (Fig. 9). It can be seen that all cycloheximide-treated cultures synthesized less viral RNA than the control, that the period of viral RNA synthesis was extended after cycloheximide treatment and that the extension was greater than the duration of cycloheximide treatment.

Another set of co-infected cultures was treated with cycloheximide at the times and for the periods shown in Table 5. After removal of cycloheximide the rate of uptake of ^{3}H-leucine into each culture was measured every 20 min for the next 6 h. Representative data are shown in Fig. 10 and relevant parameters are tabulated in Table 5. The rate of viral protein synthesis after cycloheximide treatment never exceeded 52% of the maximal rate of viral protein synthesis observed in the untreated control. In many cases, cultures recovered to produce maximal rates of viral protein synthesis which were less than 20% of the control maximum. The times of maximal rates of viral protein synthesis were delayed by cycloheximide treatment by an amount of time greater than the duration of the cycloheximide treatment. Thus following cycloheximide treatment, viral protein is synthesized at a lower than normal rate, requires a longer time to reach its maximal rate, and does not last long enough to produce a normal amount of viral protein. The implications of this data for a model of interference based on replication will be discussed below.

It should also be noted in Fig. 10 and Table 5 that those samples treated with cycloheximide at later times show better recovery than those treated earlier. A culture which was treated for 30 min at 150 min after infection recovered to

TABLE 3. *Dependence of DI particle hyperenrichment on the duration of exposure to cycloheximide*[a]

Time of addition of cycloheximide (min after infection)	Duration of treatment (min)	Distribution of progeny virus[b]	
		Standard virus (%)	DI(1) particles (%)
0	10	37	63
0	30	35	65
0	60	32	68
0	120	30	70
15	10	38	62
15	30	35	65
15	60	26	74
15	120	24	76
Untreated[c]		46	54

[a] One-third of an actinomycin D-treated culture of 4.8×10^8 cells in 120 ml, infected with a mixed stock of standard and DI(1) particles, was treated with cycloheximide (100 μg/ml) beginning at 0 min after infection for the time periods shown. One-third of the culture was similarly treated beginning 15 min after infection. One-third of the culture received no cycloheximide and was exposed to ^{14}C-uridine (40 μCi) from 60 to 360 min after infection. Removal of cycloheximide, labeling, and processing of the virus is described in the legend to Table 2.

[b] See footnote b, Table 1.

[c] See footnote c, Table 2.

synthesize viral protein at a maximal rate of 52% of the control maximal rate, whereas those cultures similarly treated at the time of infection or 20 min after infection recovered to maximal rates 18 and 42% of the control maxima, respectively.

TABLE 4. *Dependence of cycloheximide-mediated DI particle hyperenrichment on the concentration of cycloheximide*[a]

Conc of cycloheximide (μg/ml)	Distribution of progeny virus[b]	
	Standard virus (%)	DI(1) particles (%)
10	42	58
100	32	68
200	28	72
No treatment	45	55

[a] Portions of an actinomycin D-treated culture (10 ml) of 2.4×10^8 cells in 60 ml were exposed to cycloheximide for 60 min beginning at the time of infection. The other 30 ml of the culture received ^{14}C-uridine (30 μCi) from 60 to 360 min after infection.

[b] See footnote b, Table 1.

[c] See footnote c, Table 2.

Effect of cycloheximide on total particle yield. Previous experiments (3) have demonstrated that in cells infected by standard and DI particles, the relative yield of total virions (standard plus DI) is numerically almost exactly equal to the percentage of standard virus in the inoculum. Any treatment which alters this equivalence must alter the effective input ratio. If cycloheximide were acting in the above experiments to alter enrichment by differentially affecting the relative initial rates of viral replication, then the relative yield of physical particles ought to be more equal to the percentage of standard virus in the progeny than in the inoculum.

In order to compare the yields of physical particles under various conditions, four cultures of cells were infected by varying ratios of standard to DI particles. Half of each culture received cycloheximide (100 μg/ml) for 30 min beginning 20 min after infection and all cultures received ^{3}H-uridine beginning 60 min after

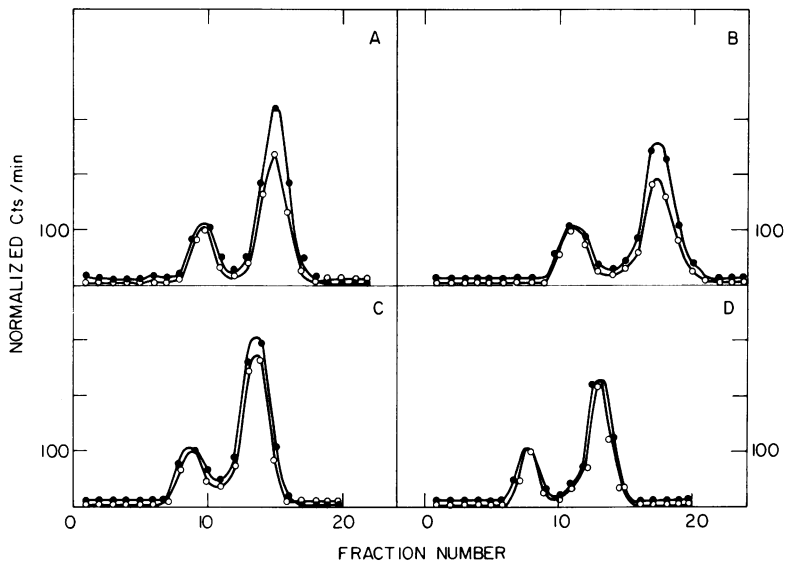

FIG. 8. *CsCl density gradients of the progeny of standard and DI particle co-infected cells showing transient hyperenrichment in virus produced following cycloheximide treatment. An actinomycin D-treated culture of 1.2 × 10⁸ cells in 30 ml was infected with a mixed stock of standard and DI(1) particles. The culture received cycloheximide (100 μg/ml) for 30 min beginning 20 min after infection. Cycloheximide was removed by washing cells twice with Earle saline and resuspending them in normal medium plus 5% horse serum. One-third of the culture received ^{14}C-uridine (10 μCi) beginning 60 min after infection and virus was harvested 420 min after infection. The other two-thirds of the culture received 2 mCi of ^{3}H-uridine (in 2 ml of medium) at 240 min after infection. Portions of this culture were removed at various times after addition of label, washed once with Earle saline, and resuspended in 1 ml of Earle saline. Virus was released by three cycles of freezing and thawing. A portion of the ^{14}C-uridine-labeled culture was added to each ^{3}H-uridine-labeled culture. Virus was examined in CsCl gradients. The data was normalized as described in the legend to Fig. 1. The duration of labeling was (A) 8 min; (B) 18 min; (C) 30 min; and (D) 60 min. Symbols: ○, ^{14}C-uridine-labeled virus; ●, ^{3}H-uridine-labeled virus.*

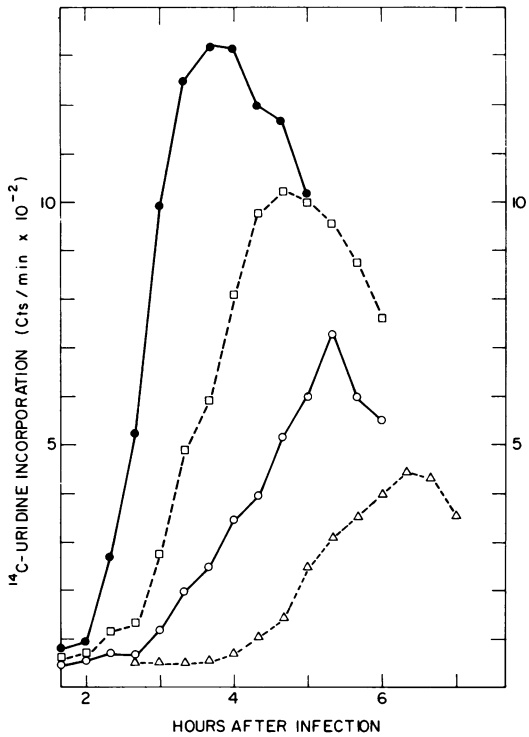

FIG. 9. *Effect of cycloheximide on the incorporation of ^{14}C-uridine into viral RNA in co-infected cells. Cultures of 10^7 actinomycin D-treated cells, infected with a mixture of standard and DI(1) particles, were treated with cycloheximide (100 μg/ml) for 10 min beginning 20 min after infection (□); 60 min beginning at the time of infection (○); or 120 min beginning 20 min after infection (△). Cycloheximide was removed by washing cells twice with Earle saline. Cells were resuspended in normal medium containing 5% horse serum. A control culture received no cycloheximide (●). ^{14}C-uridine (0.25 μCi) was added to each culture 90 min after infection or after cycloheximide removal. Every 30 min, 0.1-ml portions were withdrawn from each culture and acid-precipitable radioactivity was determined. The data has been corrected for the incorporation of ^{14}C-uridine into an actinomycin D-treated mock-infected culture.*

infection. Progeny virus was harvested 7 h after infection and mixed with a measured amount of ^{14}C-uridine-labeled standard virus-infected cells to allow correction for loss. These mixtures were processed, centrifuged to equilibrium in CsCl density gradients, and the proportion of standard and DI particles in the progeny was calculated. From the amount of ^{14}C-uridine-labeled standard virus recovered, relative total physical particle yields were computed (Table 6).

Comparing the cycloheximide-treated and untreated portions of the cultures infected at different multiplicities, the hyperenrichment produced by the drug is evident (Table 6, column C). Cycloheximide also reduced the total particle yield (Table 6, column D). However, its effect on the total yield was greater than in the control infected with standard virus alone (Table 6, column E). The effects of cycloheximide on the relative yield of standard virus and on the total yield of physical particles were approximately equal (compare columns C and E in Table 6) indicating that cycloheximide was acting as if to change the ratio of infecting particles.

DISCUSSION

The experiments presented here demonstrate that the extent of enrichment of DI particles in the progeny from cells infected by both standard and DI particles is a parameter which can be varied by numerous types of treatment of the infected cells. Hyperenrichment was observed in labeled virus produced shortly after addition of labeled uridine, in the progeny from infected cells which were shifted to temperatures below 37 C at 150 min after infection, and when co-infected cells were treated with cycloheximide. The transient hyperenrichment revealed by pulse-labeling could be observed not only in

TABLE 5. *Effect of cycloheximide treatment on incorporation of ^{14}C-uridine into viral RNA in cells co-infected by standard and DI(1) particles*[a]

Time of addition of cycloheximide (min after infection)	Duration of treatment (min)	Control maximal incorporation (%)[b]	Time of maximal incorporation (min)
No treatment	No treatment	100	170
20	10	48	230
0	30	18	260
20	30	42	240
150	30	52	210
0	60	15	290
20	60	20	230

[a] Separate 2.5-ml cultures of 10^7 actinomycin D-treated cells infected with a mixed stock of standard and DI(1) particles were treated with cycloheximide (100 μg/ml) at the times and for the periods shown. Cycloheximide was removed by washing cells twice with Earle saline. Cells were resuspended in normal medium containing 5% horse serum. ^{14}C-uridine (0.25 μCi) was added to each culture 90 min after infection or after cycloheximide was removed. Every 30 min, 0.1-ml samples were removed and incorporation of ^{14}C-uridine into acid-precipitable material was determined. Representative data from this experiment are shown graphically in Fig. 8.

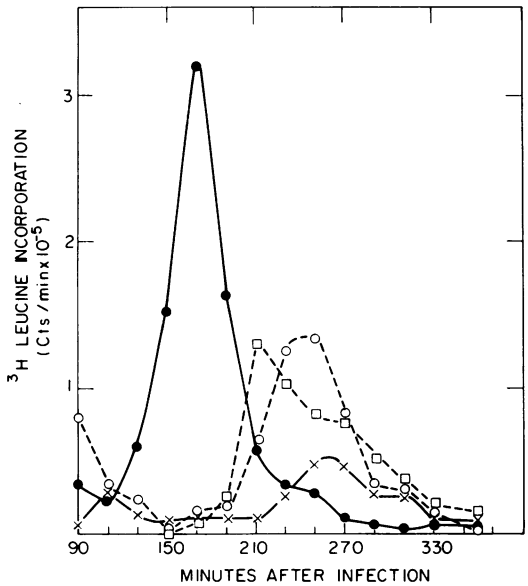

FIG. 10. *Effect of cycloheximide treatment on the rate of viral protein synthesis in co-infected cells. Cultures of 2 × 10^8 actinomycin D-treated cells, infected with a mixture of standard and DI(1) particles were treated with cycloheximide (100 μg/ml) for 30 min beginning at the time of infection (×); 20 min after infection (○); or 150 min (□). Other cultures were treated with cycloheximide for different time periods beginning at different times after infection and are listed in Table 5. After cycloheximide treatment, cells were washed twice and resuspended in Earle saline containing 5% normal medium and 5% dialyzed horse serum. A control culture received no cycloheximide and was maintained in Earle saline containing 5% normal medium and 5% dialyzed horse serum (●). Beginning 90 min after infection and continuing every 20 min up to 360 min after infection, 0.25-ml portions were removed from each culture. To each portion, 0.75 ml of Earle saline containing 25 μCi of ^{3}H-leucine was added. After 15 min, labeled cells were cooled rapidly, washed once, and resuspended in 0.5 ml of 0.01 M NaCl, 0.0015M MgCl, 0.01M Tris, pH 7.4. Each sample received 0.5 ml of 2N NaOH at room temperature for 10 min followed by 2 ml 10% trichloroacetic acid. Acid-precipitable material was collected on filters and radioactivity was determined.*

normal conditions but also as an effect superimposed on the hyperenrichment seen after temperature shift and cycloheximide treatment. Constant incubation of co-infected cultures at temperatures other than 37 C was the only treatment found to result in a relative decre-

TABLE 6. *Effect of cycloheximide treatment on the progeny distribution and total physical particle yields from cultures infected with different multiplicities of standard and DI(1) particles*[a]

(A) Multiplicity of infecting virus[b]		(B) Cycloheximide added[c]	(C) Distribution of progeny virus[d]		(D) Normalized radioactivity in total particles[e] (counts/min × 10^7)	(E) Total physical particle yield (% of control)
Standard virus	DI(1) particles		Standard virus (%)	DI(1) particles (%)		
47 (100)		−	100		6.98	100
		+	100		3.88	100
40 (67)	20 (33)	−	61	39	4.85	69
		+	51	49	1.98	51
15 (33)	30 (67)	−	29	71	2.54	36
		+	17	83	0.81	21
10 (28)	25 (72)	−	23	77	1.34	19
		+	16	84	0.52	13

[a] Cultures of 8 × 10^7 cells were infected with standard poliovirus or DI(1) particles, or both in the combinations and at the multiplicities listed.

[b] Numbers listed are multiplicities of infection. Numbers in parentheses give percentage of the inoculum as standard virus and DI(1) particles.

[c] Cycloheximide (100 μg/ml) was added to half of each culture for 30 min beginning 20 min after infection. All cultures were then washed twice with Earle saline and cells were resuspended in normal medium plus 5% horse serum.

[d] Each culture received 0.25 mCi of ^{3}H-uridine 60 min after infection. At 420 min after infection, each culture was mixed with a measured amount of a ^{14}C-uridine-labeled standard virus-infected culture, and the particles were processed as described in the legend to Fig. 2 (4). The amount of ^{3}H-uridine label in standard virus and DI(1) particles was determined and expressed as a percentage of the total progeny from each culture. These values have been corrected for the smaller genome size of DI(1) particles.

[e] The amount of ^{3}H-uridine and ^{14}C-uridine label in virus particles in each CsCl gradient was determined. The ^{3}H/^{14}C ratio was computed to correct for loss of material during processing.

The control for cycloheximide-treated cultures was the cycloheximide-treated culture infected only by standard virus. The control for untreated cultures was the untreated culture infected only by standard virus.

ment of DI particles in the progeny yield.

These results are difficult to explain by any unitary mechanism for altering enrichment. Rather, it appears that two separate mechanisms of enrichment, preferential replication and preferential encapsidation, are needed to explain the results. We will first develop the theory of enrichment by these two mechanisms and then consider how they relate to our experimental results.

Mechanisms of enrichment. The concept of enrichment occurring by preferential replication involves the hypothesis that a shorter RNA is able to replicate more extensively per unit time than a larger RNA. As applied to our poliovirus system, DI RNA could be enriched by replicating more efficiently than standard RNA.

One way that DI RNA could replicate more efficiently than standard RNA would be for it to have a different and more efficient initiation site for RNA synthesis than standard RNA. This mechanism does not appear to relate to the poliovirus system because it would produce a continual acceleration of enrichment as the infection cycle progresses which is an effect we have not observed (3).

If two RNAs have the same initiation site, in order for a shorter molecule to have a replicative advantage over a longer one, one condition must be met: the amount of viral RNA polymerase in the infected cell must be the limiting factor in the replication process. In principle, the rate of RNA synthesis could be limited either by the availability of initiation sites for replication or by the amount of polymerase. When initiation sites are limiting, then the number of molecules formed is independent of the length of the molecule and a shorter RNA will not be enriched. Only when the concentration of polymerase molecules limits the rate of RNA synthesis will a shorter RNA have a replicative advantage. That advantage will come about because a polymerase molecule will be released from the shorter replicative intermediate sooner after initiation than a polymerase molecule involved in standard RNA synthesis (this assumes that elongation and termination rates are the same for the two RNAs).

The second possible mechanism of enrichment, enrichment due to preferential encapsidation of DI RNA, would involve an affinity between capsid protein and DI RNA greater than that between capsid protein and standard RNA. Such an increased affinity could occur due to the creation of an altered binding site for capsid protein on the DI RNA. Standard virus might be restricted from acquiring such a high affinity binding site if it involved the deletion of essential genetic information.

Interpretation of results. The different manipulations and treatments studied here which led to altered enrichment can each be most easily attributed either to the process of preferential replication or to the process of preferential encapsidation:

(i) Transient hyperenrichment, occurring either alone or in combination with another hyperenrichment, would appear to be due to preferential encapsidation. This transient effect implies a more rapid rate of formation of DI particles than of standard particles and therefore a higher affinity of capsid protein for DI RNA than for standard RNA. If the procapsid is the form of capsid protein which interacts with viral RNA (6, 7), then our experiments indicate a higher affinity of procapsid for DI RNA than for standard RNA.

(ii) Hyperenrichment resulting from a temperature downshift is probably also due to an effect on encapsidation. Since standard and DI RNA synthesis proceed at similar reduced levels after temperature shift, a relative change in RNA synthesis does not seem to be involved in this phenomenon. There is apparently an alteration in the affinity between procapsid and standard or DI RNA caused by lowering the temperature. This interpretation also suggests that a higher affinity of protein for viral RNA can lead to a changed equilibrium level of enrichment because the change induced by lowering the temperature affects all of the particles made thereafter.

(iii) The experiments utilizing cycloheximide can most easily be explained as affecting replication. These experiments were originally carried out in an attempt to make viral RNA polymerase more limiting. That this actually happens is suggested by the findings that following removal of cycloheximide, the exponential phase of viral RNA synthesis (1) is extended and the amount and rate of viral protein synthesis are reduced (Fig. 8 and 9). Cycloheximide might also be acting to allow previously made limiting polymerase to continue RNA synthesis because treatment with cycloheximide for one hour beginning anytime in the first 30 min of the infection cycle led to greater enrichment than treatment at the time of infection (Table 2).

In addition, the experiments presented in Table 6 demonstrate that cycloheximide treatment results in an alteration of the effective input ratio of standard to defective particles as measured by the yield of total physical particles. This is similar to the effect produced by delaying the addition of standard virus to DI particle-infected cultures (3). This result of cycloheximide treatment is consistent with the

idea that a period of time in the presence of cycloheximide leads to an alteration in the population of replicating molecules rather than a change in their rate of encapsidation.

(iv) An effect which is probably unrelated to the others is the decrement in DI particles observed on incubation of co-infected cultures continuously at temperatures other than 37 C. These results appear to be due to the only known defect in DI particle function for RNA synthesis, a defect in early RNA synthesis at low temperatures. The reason for the effect is unclear but it is in contrast to our earlier findings that DI RNA synthesis was completely normal at 37 C (2).

Mechanism of enrichment in the unperturbed infection cycle. Finally, we wish to be able to explain the mechanism(s) responsible for the normal equilibrium enrichment observed in co-infected cultures maintained continuously at 37 C. Since the extent of enrichment is only 5 to 8% in any single infection cycle, experiments of the type presented in Table 6, seeking to establish whether the relative total particle yield is related more closely to the percentage of standard virus in the inoculum or in the progeny, lack the precision necessary to give an answer (3). A case can be made, however, that both mechanisms we have considered above are operative.

The involvement of preferential replication of DI RNA in the enrichment process is very likely since the only precondition for its occurrence, limiting amounts of viral RNA polymerase, probably occurs early during the infection cycle. The cycloheximide effect could then be considered as a return to and lengthening of the conditions pertaining during the earliest stages of the infection.

Preferential encapsidation probably also plays a role in normal equilibrium enrichment. The transient hyperenrichment visualized by pulse-labeling, which is a reflection of differential rates of encapsidation, is also likely to reflect an equilibrium enrichment of DI particles. The ability of a downshift of temperature to produce a changed equilibrium enrichment strongly suggests that the normal transient hyperenrichment has its counterpart in the normal equilibrium enrichment.

The mechanism of enrichment appears, thus, to involve both replication and encapsidation. In the co-infected cell, the needed conditions seem to be met for each to contribute to the observed enrichment. As enrichment in other animal virus DI particle systems is studied, different contributions of these two processes may be revealed.

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