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Kinetics of Incorporation of Structural Proteins into Sindbis Virions

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The morphogenesis of Sindbis virus was studied by determining the kinetics with which newly synthesized nucleocapsid and envelope proteins appeared in virions released into the extracellular medium. Assembly of the nucleocapsid was more rapid than modification of the cellular membrane by the addition of the viral envelope protein. However, both viral structural proteins were efficiently incorporated into virions; a 0.5-hr pulse-labeling period resulted in the release of maximally labeled virus during the next hour. When protein synthesis was inhibited, release of virus soon declined even though large amounts of both viral structural proteins were present within the cell and ribonucleic acid replication was unaffected.

At present, little is known about the morphogenesis of arboviruses or any other animal virus; yet the maturation of Sindbis virus, a group A arbovirus, must be relatively simple because the virion itself contains only two virus-determined proteins, one in the nucleocapsid and one in the envelope (12). The nucleocapsid of Sindbis virus acquires its lipoprotein envelope by budding through a cellular membrane that has been altered by the addition of viral envelope protein. Since this mode of release does not grossly damage the infected cell, virus is produced at a rapid, nearly constant rate for several hours (10). Viral morphogenesis during this period requires three principal steps: (i) assembly of the nucleocapsid, (ii) insertion of the envelope protein into cellular membrane, and (iii) release of virions as enveloped nucleocapsids.

Temperature-sensitive (TS) mutants (3, 5) defective in viral structural proteins are potentially valuable for the analysis of each of these steps. When cultures infected by such mutants are shifted to a nonpermissive temperature, further production of functional protein molecules corresponding to the particular lesion of the mutant should cease. The resulting deficiency should disturb viral maturation in an identifiable manner. However, to interpret studies with mutants, it is necessary to understand certain basic parameters of the morphogenesis of wild-type virus. We report here kinetic studies on the utilization of the nucleocapsid and envelope proteins in the formation of Sindbis virus.

We have also examined the production of virus by cultures in which protein synthesis was inhibited during the course of infection. This inhibition might mimic the effect of shifting cultures infected by a *ts* mutant to the nonpermissive temperature, although, of course, in the case of the mutant the synthesis of functional protein corresponding to only one viral cistron would cease.

MATERIALS AND METHODS

Tissue culture and viruses. Methods for the growth and titration of Sindbis virus in primary chick embryo fibroblast cultures have been described (10). The HR strain of Sindbis virus (3) was used in all experiments. Although this strain has no *ts* mutations, it differs from wild-type virus in being more resistant to heat inactivation at 60 C.

Isotopic labeling of virus. Cultures with 10 plaque-forming units (PFU) adsorbed per cell were incubated at 37 C with Eagle's medium containing 1 μg of actinomycin D per ml (Merck & Co., Inc., Rahway, N.J.) and 3% rabbit serum. To obtain uniformly labeled virus, the appropriate isotope (^{3}H-uridine, ^{14}C-reconstituted protein hydrolysate, or ^{3}H-leucine; Schwarz BioResearch, Inc., Orangeburg, N.Y.) in fresh prewarmed medium was added 2 to 3 hr after infection. The medium containing the released virus was harvested 6 to 8 hr later.

When virus pulse-labeled with ^{3}H-leucine was desired, the monolayers were infected as above except that the amount of leucine present was reduced to 1 μg/ml. At a specific time, medium containing radioactive leucine was added. After exposure to the isotope, the cultures were washed with medium supplemented with four times the normal quantity of leucine. Any further incubation was performed in this chase medium.

Single-step purification of isotopically labeled virus. Experiments designed to study the kinetics of the in-

corporation of viral proteins into virions required the purification of many small samples of virus. Since previously reported methods for the purification of Sindbis virus (10, 12) were unsuitable for multiple small samples, we devised an alternative procedure that combined some of the features of velocity and isopycnic centrifugation. To form a 20-ml gradient, 1 ml of 50% sucrose in 0.2 M CsCl and 0.002 M tris(hydroxymethyl)aminomethane (Tris, *p*H 7.8) was first added to the bottom of the tube. Next, a linear density gradient (1.127 to 1.245 g/cm^3) was prepared with 3.0 ml of 25% sucrose (w/w) in phosphate-buffered saline (PBS; 8), lacking calcium and magnesium, and 3.0 ml of 50% sucrose in 0.2 M CsCl and 0.002 M Tris. Sindbis virus forms an isopycnic band in this gradient. On top of this density gradient, a 13-ml linear gradient was constructed with 5 and 20% (w/w) sucrose in PBS. Rabbit serum (final concentration, 0.1%) was present throughout the gradient to stabilize infectivity. Lastly, a 5-ml sample of medium was carefully layered over the prepared 20-ml gradient. Centrifugation was for 3.5 hr at 4 C in a Spinco SW 25 rotor at 25,000 rev/min. Fractions were collected by puncturing the tube and collecting 25-drop samples. These samples were diluted with 0.1 M sodium phosphate buffer (*p*H 7.2) to a specified volume. Bray's scintillation solution (2) was added to a portion of each fraction for determination of radioactivity. The fractions containing the desired radioactive viral peak were pooled, further diluted to 9 ml with 0.1 M phosphate buffer (*p*H 7.2), and centrifuged in a Spinco angle-head rotor at 30,000 rev/min, for 45 min at 4 C. The nearly invisible viral pellet was prepared for electrophoresis by treatment with 0.2 to 0.3 ml of 1% sodium dodecyl sulfate (SDS) and 1% mercaptoethanol in 0.1 M phosphate buffer (*p*H 7.2) at 37 C for 30 min. This material was stored at −70 C for subsequent electrophoretic analysis.

Preparation of cell extracts. The monolayers were washed several times with PBS and then dissolved in 1 ml of 0.1 M phosphate buffer (*p*H 7.2) containing 1% SDS and 1% mercaptoethanol. After heating at 37 C for 30 min, the cell extracts were dialyzed overnight against 0.01 M phosphate buffer (*p*H 7.2) containing 0.1% SDS and 0.1% mercaptoethanol with two changes of buffer.

Polyacrylamide-gel electrophoresis. Proteins from purified virus and cell extracts were solubilized as above and mixed with 1/6 volume 60% sucrose. Polyacrylamide gels (10%) were prepared by the method of Summers et al. (13). Electrophoresis was performed in glass tubes (0.7 by 27.5 cm) on vertical 18-cm gel columns at 5 v/cm for 15 hr at room temperature. The gels were sliced into 6-cm lengths and manually inserted into a 1-cc tuberculin syringe fitted with the hub of a 21-gauge needle. The gel was pulverized by extrusion of 0.1-ml fractions into glass scintillation vials containing 1 ml of water. The samples remained at room temperature overnight. Finally, Bray's scintillation solution (2) was added and the radioactivity of each fraction was measured.

Measurement of total acid-insoluble radioactivity. Monolayers were washed with PBS and then scraped into 1 ml of 0.1 M phosphate buffer (*p*H 7.2). A portion of each sample was solubilized for electrophoresis. The remainder was precipitated with 0.3 N trichloroacetic acid in the presence of 1 mg of cold leucine. After about 16 hr at 4 C, the samples were centrifuged in the cold and the pellets were washed twice with 0.3 N trichloroacetic acid. The supernatant fluid was diluted 1:10 in 0.3 N NH_4OH, and the radioactivity of the acid-soluble portion was determined. The acid-insoluble material in the pellet was treated with 0.5 ml of 1 N NaOH and diluted 1:100 in 0.3 N NH_4OH for measurement of radioactivity.

A sample of the culture fluid removed from the above monolayers was treated in the manner just described. The rest of the medium was purified by the single-step procedure to permit analysis of acid-insoluble material present in virus.

Sedimentation analysis of isotopically labeled viral ribonucleic acid (RNA). Actinomycin D-resistant ^{3}H-uridine incorporation was measured as described previously (4). Analysis of labeled viral (RNA by sedimentation through a 15 to 30% glycerol-SDS gradient has been reported (4).

Hemagglutination. The capacity of Sindbis virus to hemagglutinate goose redblood cells was determined by the technique of Clarke and Casals (6).

RESULTS

Purification of isotopically labeled virus. The purification of isotopically labeled virus obtained by our single-step procedure is illustrated in Fig. 1. Viral RNA, labeled protein, and viral infectivity all banded in the same isopycnic region of the gradient. Moreover, the virus was not contaminated with low-molecular-weight precursors from the radioactive medium. Further analysis of virus prepared by this method revealed it to be homogenous by several criteria (10). Since this method was divised to prepare virus for subsequent analysis of labeled proteins by acrylamide gel electrophoresis, such an analysis may perhaps best demonstrate the purification attained. Figure 2A shows the electropherogram of virus labeled with ^{14}C-amino acids. Our results were identical to those obtained by Strauss et al. (12), who used a more elaborate method of purification. Only two labeled proteins were found. The peak of greater mobility, identified previously (12) as the nucleocapsid protein, contained 24% of the radioactivity; the peak of lesser mobility, the envelope protein, contained 76%. Strauss et al. (12) reported 25% and 75%, respectively.

Since most of our studies used ^{3}H-leucine rather than ^{14}C-protein hydrolysate, the electrophoretic pattern of virus uniformly labeled in this manner was also determined. Figure 2B shows that again only two labeled peaks were found, although the distribution of radioactivity was not the same. In leucine-labeled virus, 84% of the radioactivity was in envelope protein, whereas 16% was in nucleocapsid protein. This

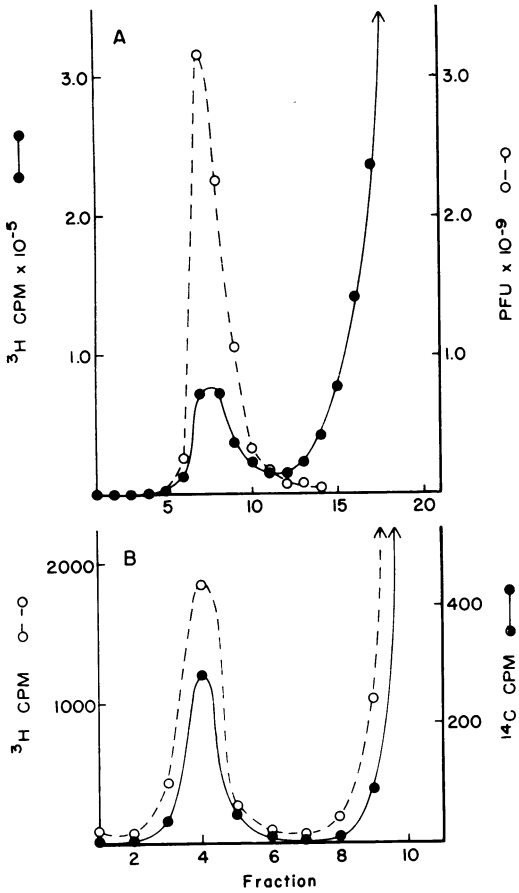

FIG. 1. *Single-step purification of isotopically labeled Sindbis virus. Monolayers were infected and incubated with ^{3}H-leucine (6 c/mmole, 50 μc/ml) or a mixture of ^{3}H-uridine (20 c/mmole, 125 μc/ml) and ^{14}C-reconstituted protein hydrolysate (12.5 μc/ml), as described in Materials and Methods. The uniformly labeled virus released into the medium was purified by the single-step procedure. Sedimentation was from right to left. (A) Association of ^{3}H-leucine-labeled protein with infectivity. (B) Association of ^{3}H-uridine virus-specific RNA with ^{14}C-amino acid-labeled protein.*

difference between virus labeled with ^{14}C-protein hydrolysate and ^{3}H-leucine is probably a reflection of the amino acid composition of the nucleocapsid and envelope proteins. It is not surprising that the envelope protein should be relatively rich in a hydrophobic amino acid such as leucine.

Kinetics of ^{3}H-leucine incorporation into Sindbis virus. We used a pulse-labeling technique to establish the minimal time for the formation of Sindbis virus. Cells infected for 4 hr were exposed to ^{3}H-leucine for 0.5 hr and then further incubated in chase medium which was changed every 0.5 hr. The specific radioactivity of the virus (counts/min per PFU) in each serial sample of medium was determined (Fig. 3). Virus released during the 30-min labeling period was slightly radioactive, but the most radioactive virus was found in the hour immediately after the pulse. Virus released in subsequent intervals was progressively less radioactive. A similar pattern was obtained in cultures pulse-labeled later in the course of infection (Fig. 4), although the maximal specific radioactivity was only 50% that of the earlier labeling period.

The observation that virus containing the highest radioactivity was released shortly after the 0.5-hr pulse with ^{3}H-leucine could be explained by at least two models. The formation of virus could be a steady state in which no surplus viral protein was produced and all viral structural proteins were rapidly incorporated into virions released into the medium. Alternatively, viral protein could be made in excess of that needed for the observed viral release, and the pool of viral precursor protein could be constantly ex-

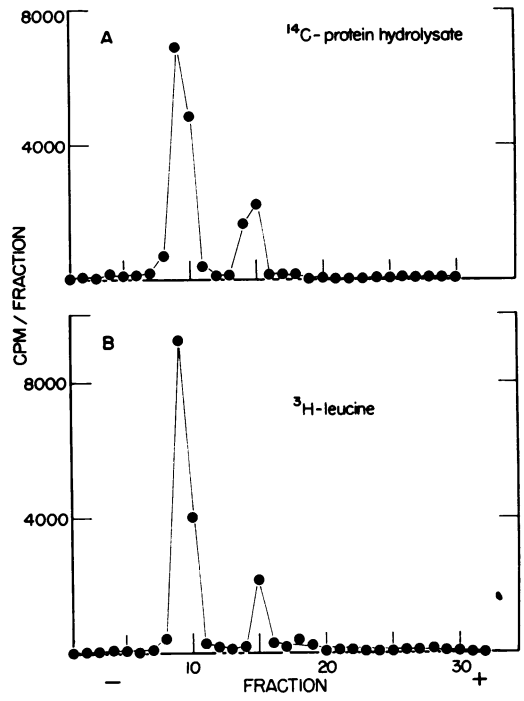

FIG. 2. *Acrylamide-gel electrophoresis of purified, isotopically labeled Sindbis virus. Virus was uniformly labeled, purified, and subjected to electrophoresis as described in Materials and Methods. Samples of 60 μliters were applied to the gels. (A) Virus labeled with ^{14}C-reconstituted protein hydrolysate (3.3 μc/ml). (B) Virus labeled with ^{3}H-leucine (6c/mmole, 50 μc/ml).*

panding. The first model predicts that virus released within a constant interval after pulses given any time during release should have the same specific radioactivity, since there is no significant pool of unlabeled precursor protein available to dilute newly synthesized radioactive protein. Conversely, the second theory predicts that progressively later pulses should yield virus of lower specific radioactivity because the expanding pool dilutes newly synthesized radioactive viral protein. These models were tested by pulse-labeling a series of replicate cultures for various 0.5-hr intervals and determining the specific radioactivity of virus released during the hour immediately after the pulse. The specific activity of the virus declined steadily as the labeling was performed later and later (Fig. 5). This decrease was probably not due to a change in the PFU to particle ratio since the PFU to hemagglutinating activity ratio of the various samples showed no trend or significant difference.

A direct test of the expanding-pool model would be the demonstration that substantial quantities of viral structural proteins synthesized early in the course of infection remain in the cell and are not incorporated into virions. This prediction was confirmed by an experiment in which a series of replicate cultures were pulse-labeled from 4 to 4.5 hr with ^{3}H-leucine. Immediately after the pulse, cell samples were taken. The remaining cultures were incubated for an additional 4 hr in chase medium. At 8.5 hr, both the culture

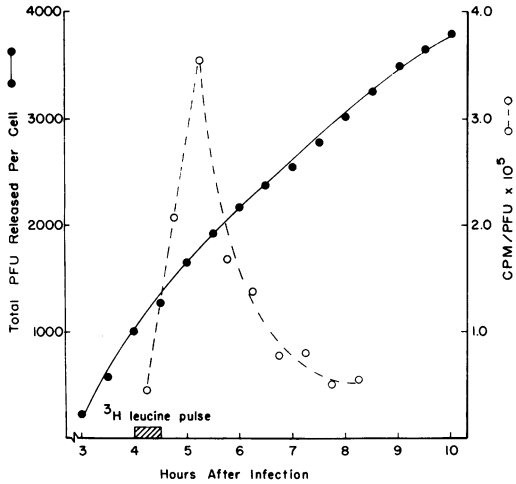

FIG. 3. *Kinetics of ^{3}H-leucine incorporation into Sindbis virus. Cultures with 10 PFU adsorbed per cell were incubated at 37 C for 4 hr in medium containing 1 μg of leucine per ml, 1 μg of actinomycin D per ml, and 3% rabbit serum. At 4 hr, the monolayers were washed and pulse-labeled with ^{3}H-leucine (6 c/mmole, 50 μc/ml) for 0.5 hr. After exposure to the isotope, the cells were washed with chase medium containing 100 μg of leucine per ml. Thereafter, the chase medium was changed at 0.5-hr intervals. The culture fluids were purified by the single-step procedure and analyzed for infectivity and radioactivity.*

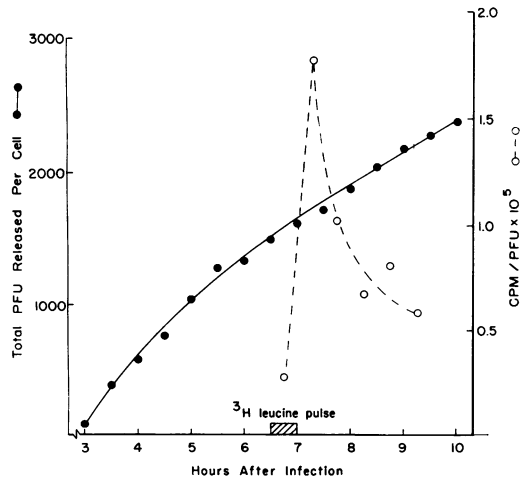

FIG. 4. *Kinetics of ^{3}H-leucine incorporation into Sindbis virus. Procedure was identical to Fig. 3, except that the 0.5-hr pulse was administered between 6.5 and 7 hr.*

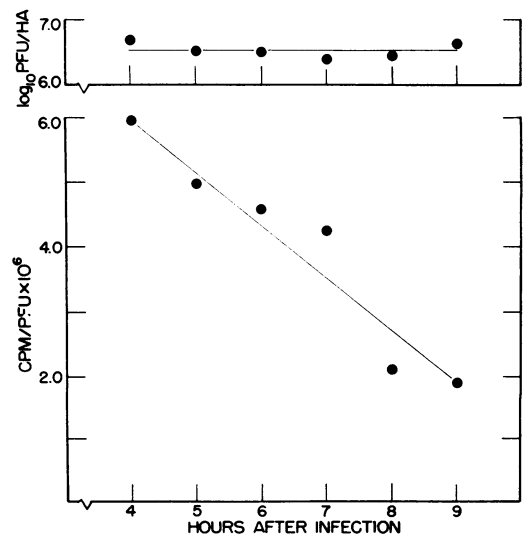

FIG. 5. *Kinetics of ^{3}H-leucine incorporation into Sindbis virus. Replicate cultures were infected as described in the legend of Fig. 3. At specified times, the cells were pulse-labeled with ^{3}H-leucine (4 c/mmole, 50 μc/ml) for 0.5 hr. After an additional hour of incubation in chase medium, the culture fluid was assayed for hemagglutinating activity, radioactivity, and infectivity.*

fluid and the rest of the monolayers were harvested for analysis.

Figure 6 shows the electrophoretic patterns of the early and late cell samples and of the virus released between 4.5 and 8.5 hr. The electropherograms have been corrected so that each represents the total radioactivity in its respective sample. As noted by Strauss et al. (12), the radioactive peaks corresponding to the viral structural proteins have no counterpart in uninfected cells and, thus, probably represent true viral proteins.

It is clear that more of the viral structural protein synthesized 4 to 4.5 hr after infection was present in the cells 4 hr later than was present in the virus released over the same interval.

The decrease in total labeled protein in the monolayer during the 4-hr chase was probably due to loss of cells by lysis or detachment late in the infection, since most of this labeled protein was found in the culture fluid.

Distribution of ^{3}H-leucine incorporated into nucleocapsid and envelope proteins of Sindbis virus. Although much of the virus-determined protein remained within the infected cell, some (Fig. 3) was rapidly incorporated into virions. We examined this process of viral morphogenesis in more detail by determining the kinetics of appearance of both viral proteins in the newly released progeny. Purified virus samples from the pulse-labeling studies of Fig. 3 were analyzed by acrylamide-gel electrophoresis, and the distribution of ^{3}H-leucine between the two viral proteins was calculated.

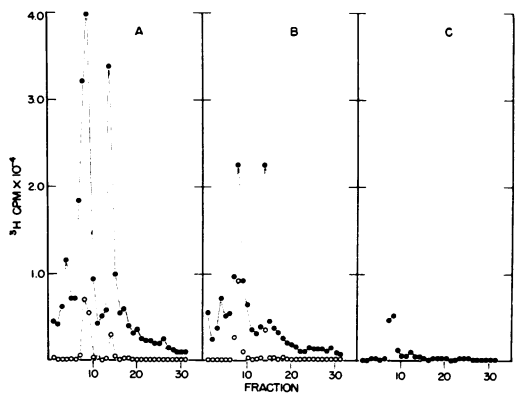

FIG. 6. *Distribution of total acid-insoluble radioactivity associated with Sindbis-infected cultures. Replicate cultures were infected as described in the legend of Fig. 3. At 4 hr, the cells were pulse-labeled with ^{3}H-leucine (4 c/mmole, 25 μc/ml) for 0.5 hr. Immediately after the pulse, 50% of the cultures were harvested as described in Materials and Methods. The remaining cells were incubated in chase medium for an additional 4 hr and then harvested. The harvested monolayers and the medium containing virus released between 4.5 and 8.5 hr were analyzed for the total quantity and distribution of acid-insoluble radioactivity. Values from the electropherogram have been corrected to represent the total acid-insoluble radioactivity present in the sample. The nucleocapsid and envelope proteins are identifiable by the ^{14}C-marker (○). (A) Infected cells at 4.5 hr. (B) Infected cells at 8.5 hr. (C) Virus released between 4.5 and 8.5 hr.*

This pulse-labeling procedure yielded the same pattern of viral radioactivity as long-term labeling. That is, the radioactivity of the total virus released during and after the pulse had the same distribution of ^{3}H-leucine between the two structural proteins (Table 1) as that found in uniformly labeled virus. However, the individual samples differed markedly from uniformly labeled virus in two aspects. (i) The earliest sample of virus, that released during the 0.5-hr pulse, showed a preferential labeling of the nucleocapsid protein. This phenomenon was amplified by reducing the pulse-labeling period (^{3}H-leucine, 40 c/mmole; 305 μc/ml) to 10 min and changing the medium at subsequent 10-min intervals. The first radioactive virus was released during the 10 min following the pulse, and the nucleocapsid protein of this virus sample contained 72% of the radioactive leucine. (ii) A return to the pattern of preferential labeling of the nucleocapsid protein was seen in the viral samples of low specific radioactivity released long after the pulse. The significance of this trend will be considered in the discussion.

TABLE 1. *Distribution of radioactivity between the nucleocapsid and envelope proteins of virus released after a pulse of ^{3}H-leucine from 4 to 4.5 hr after infection*

Interval of viral release (hr)	Percentage of radioactivity in[a]	
	Nucleocapsid protein	Envelope protein
4 to 4.5	43	51
4.5 to 5	14	86
5 to 5.5	12	88
5.5 to 6	18	82
6 to 6.5	21	79
6.5 to 7	25	75
4 to 7[b]	16	84
Uniformly labeled virus (Fig. 3)	16	84

[a] Determined by acrylamide-gel electrophoresis of purified virus released during the indicated intervals.

[b] The radioactivities of the proteins from each interval were added together, and then the percentage was calculated.

Effect of inhibition of protein synthesis on viral release. Since the infected cells apparently contained a surplus of viral structural proteins, we wished to know whether viral morphogenesis could continue in the absence of further protein synthesis. To answer this question, protein synthesis was blocked with two inhibitors, puromycin and cycloheximide. When these drugs were added at any time during the phase of active viral production, subsequent release of virus was markedly inhibited after only 0.5 hr (Fig. 7). Although unable to produce virus, the drug-treated cells nonetheless contained large quantities of both viral structural proteins. Of course, one additional component of the virion, the RNA, remains to be considered. Once rapid, viral RNA replication was underway, inhibition of protein synthesis had no effect on the incorporation of ^{3}H-uridine in the presence of actinomycin D (Fig. 8B). Moreover, analysis of the viral RNA produced in inhibited cells showed a normal amount of the 42*S* RNA, the species actually present in the virion (4). Hence, it seems unlikely that the observed decrease in viral maturation was due to a cessation of viral RNA formation subsequent to inhibition of protein synthesis.

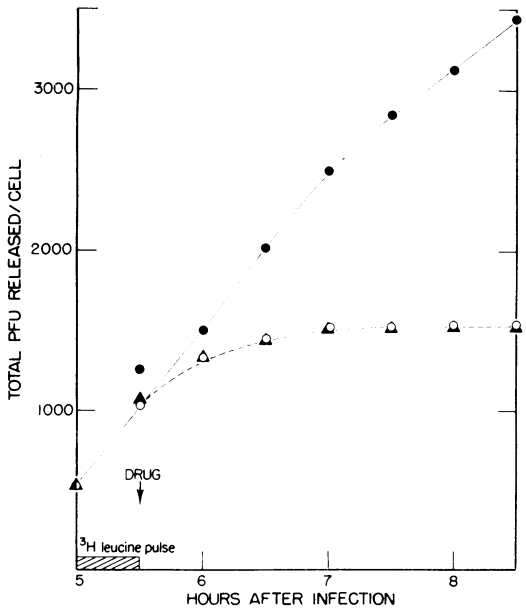

FIG. 7. *Effect of inhibition of protein synthesis on the release of Sindbis virus. Cultures were infected as described in the legend of Fig. 3. At 5 hr, the cells were pulse-labeled with ^{3}H-leucine (2 c/mmole, 25 μc/ml) for 0.5 hr. Further incubation was performed in normal chase medium (●) or chase medium containing 50 μg of puromycin/ml (▲) or 10 μg of cycloheximide/ml (○).*

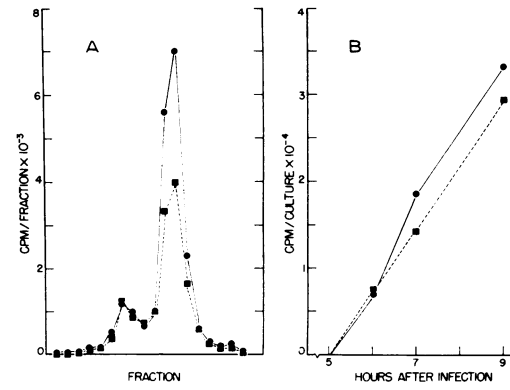

FIG. 8. *Effect of cycloheximide on the synthesis of virus-specific RNA. Infected cultures were incubated in a medium containing actinomycin D (1 μg/ml) for 5 hr and then 50% of them were treated with 10 μg/ml of cycloheximide (●); the others served as controls (■). (A) Sedimentation analysis of the virus-specific RNA. Drug-treated and control cultures were pulse-labeled with ^{3}H-uridine (20 μc/ml) for 40 min beginning 6 hr after infection. (B) Kinetics of virus-specific RNA synthesis. Drug-treated and control cultures were labeled continuously from 5 hr after infection with ^{3}H-uridine (3.3 mc/mmole, 1 μc/ml) and removed at the indicated intervals for determination of labeled RNA.*

DISCUSSION

We have examined the morphogenesis of Sindbis virus by analyzing the kinetics with which newly synthesized viral proteins appear in virions released into the extracellular medium. Our task was simplified by the observation (12) that the Sindbis virion contains only two virus-determined proteins, one in the nucleocapsid and the other in the envelope. The first isotopically labeled virus released after a short pulse of leucine showed preferential labeling of the nucleocapsid protein. Since the estimated molecular weights of these proteins (Strauss et al., *personal communication*) are of the same order of magnitude (53,000 for the envelope protein and 30,000 for the nucleocapsid protein), the time required to synthesize the respective polypeptide chains should not differ by more than 1 min (7). Thus, it appears that slightly less time is required for nucleocapsid assembly than for modification of the cellular membrane by incorporation of the viral envelope protein. However, this difference is minor and the more striking conclusion to be drawn from the kinetic data is the rapidity with which both structural proteins are incorporated into virions. In less than 20 min, a labeled amino acid added to the medium can be found in newly released virus. This speed is consistent with other reports on the formation of group A arboviruses. Intra-

cellular nucleocapsids are assembled within 3 min in cells infected by Semliki Forest virus (9) or within 10 to 15 min in cells infected by Sindbis virus (1). The time required for release of complete, infectious WEE virus particles is less than 1 min (11).

The production of Sindbis virus at a nearly constant rate for 5 to 6 hr might, at first glance, indicate a steady state in which structural proteins are utilized for virion formation as soon as they are made. We have, however, presented both direct and indirect evidence that substantially greater quantities of both viral structural proteins are synthesized than actually appear in viral particles. This surplus affects the kinetics of incorporation of newly synthesized viral proteins into virions. Nucleocapsid and envelope proteins produced late in the course of infection have a lower probability of entering virions because they compete with the preexisting structural proteins synthesized earlier in the infection.

Viral radioactivity reaches a maximum in particles released in the hour immediately following as ^{3}H-leucine pulse of 30 min. Thereafter, the radioactivity declines, and its distribution between the nucleocapsid and envelope proteins changes. The latter is progressively less efficiently labeled in virus emerging more than 1 hr after a pulse. Presently, we have no conclusive explanation of this trend. However, an unpublished observation of B. W. Burge (*personal communication*) may be relevant here. He has found that envelope protein is very efficiently incorporated into the membrane fraction of cells since there is no pool of soluble envelope protein. Some molecules of this protein may enter intracellular membranes that are not destined for viral maturation and thus be removed from the precursor pool and unavailable for viral morphogenesis. In contrast, the capsid protein, whether free or in the form of intracellular nucleocapsids, is always potentially available for viral production.

Our studies with puromycin and cycloheximide indicate that continuous protein synthesis is required for viral maturation. Even though RNA replication is not grossly affected and viral structural proteins are present within the cell, viral release is inhibited. There are several possible explanations for this phenomenon. Concomitant protein synthesis may be required to maintain the infectivity of the newly synthesized viral RNA. Alternatively, some minor structural protein or a protein involved in the assembly process may be labile and require constant synthesis. If a deficiency of only one of the virus-determined proteins is responsible for the prompt cessation of viral production in cells treated with cycloheximide or puromycin, the same pattern should be seen when cells infected by an appropriately marked *ts* mutant are shifted to the nonpermissive temperature.

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