Replication of Western Equine Encephalomyelitis Virus

II. Cytoplasmic Structure Involved in the Synthesis and Development of the Virions

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Analysis of the cytoplasmic fraction of chick embryo cells during the exponential phase of Western equine encephalomyelitis (WEE) virus growth showed that the viral ribonucleic acid (RNA) labeled by a short pulse with ³H-uridine was associated with a structure which sedimented in sucrose density gradients with a coefficient of 65S. The RNA extracted from this structure sedimented in sucrose density gradients at 26S. After a longer period of exposure to ³H-uridine, the radioactive viral RNA was associated with a structure which sedimented in sucrose density gradients as would materials with coefficients of about 140S. The 140S structure contained viral RNA and viral protein. It was shown that the 140S structures are not virus-induced polysomes. The 140S structure contained predominantly the 40S type of viral RNA and some 26S type. Electrophoretic analysis of the disrupted virion revealed that at least two proteins (types I and II) were present in the purified virion. Only type II protein was present in the 140S structure. Unlike the virion, the 140S structure did not contain any lipid which could be detected by the incorporation of ¹⁴C-choline. These data suggest that the 140S structure represents the internal nucleoprotein part of the virion. The rate of appearance of labeled virus lags behind that of the formation of the 140S structure in infected cells. Pulse-chase experiments with ³H-leucine suggest that the 140S structure may represent a precursor to the virus particle. The results are discussed in terms of the maturation of WEE virus in the infected cells.

Western equine encephalomyelitis (WEE) virus is a ribonucleic acid (RNA)-containing virus belonging to the group A arboviruses. Examination under the electron microscope has revealed that, like other group A arboviruses, the virions are spherical (diameter about 50 nm) and have a dense internal core surrounded by a membrane (4, 11). Morgan, Howe, and Rose (4) studied the development of WEE virus in chick embryo (CE) cells by using the electron microscope. They observed aggregates of viral nucleoids around the membrane-bound vacuoles in the infected cells and concluded that mature virus was formed by the budding of these nucleoids or "precursor particles" across the cellular membranes. They suggested that the aggregates of the viral nucleoids around the vacuoles may implicate the periphery of the vacuole as the site of assembly of the

nucleoids. Recently, Acheson and Tamm (1) reported essentially similar observations on the development of Semliki Forest virus (SFV), another group A arbovirus. However, they pointed out that the aggregates of the nucleoids found around the membrane-bound vacuoles may not denote the site of assembly of the nucleoids, since they did not begin to appear until late in the course of the production of infectious virus.

The role of these nucleoids in the development of the arboviruses has been further delineated by studies involving the analysis of the cytoplasmic extracts of the infected cells. Wecker and Richter (11) reported that infectious WEE viral RNA may be associated with some material which sedimented on sucrose gradients at a rate intermediate between those of single ribosomes and intact WEE virus. Incubation of the virion with sodium deoxycholate produced a structure similar in its sedimentation rate to those found in the cytoplasmic fraction. The authors concluded that

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in the infected cells the viral RNA was contained in a nucleoprotein, possibly the nucleoprotein core of the virion. However, they reported later that their observations and conclusions were erroneous. More recently, Friedman and Berezesky (3) reported the presence of two virus-specific structures containing viral RNA in the cytoplasm of cells infected with SFV. These structures sedimented slower than the virions on sucrose gradients and possessed peak sedimentation rates of 140S and 65S. The 140S structure contained RNA with a sedimentation coefficient of 42S, the same type of RNA found in virions. However, the 65S structure contained RNA with a sedimentation coefficient of 26S, one of the types of viral RNA found in cells infected with group A arboviruses. Electron micrographs of the 140S structure revealed its morphological similarity to the viral nucleoids found in the infected cells. Very recently, Strauss et al. (8) reported that the RNA-containing inner core of Sindbis virus, another group A arbovirus, can be isolated by incubating the virion with deoxycholate. The structure isolated from the virion after incubation with deoxycholate possessed a sedimentation rate of 140S on sucrose gradients. Electrophoretic analysis of the 140S structure with polyacrylamide gel revealed that it contained only one of the two types of proteins found in the virions. Thus, the 140S structure consisted of viral RNA and one type of viral protein.

The present report deals with the analysis of the cytoplasmic extracts from cells infected with WEE virus. The results show that the infected CE cells contain viral nucleoids similar to those of SFV. The composition of the 140S structure isolated from the cytoplasmic extract shows that they contain both viral RNA and one of the viral proteins. Our data indicate that the 140S structure is the viral core similar to that isolated by incubation of virions with deoxycholate. The role of this core in the development of the WEE virus is discussed.

MATERIALS AND METHODS

Cells and virus. The media used and the method of preparing CE fibroblast cultures have been described earlier (6). The source and the preparation of WEE virus and the method used to infect the cultures were identical to those reported earlier (6).

Chemicals and isotopes. Actinomycin D was a generous gift from Merck Sharp & Dohme. Isotopically labeled amino acids and uridine were purchased from Nuclear-Chicago Corp., Des Plaines, Ill.

The methods used to infect the cells and to label the viral RNA or protein were identical to those reported previously (6). Unless described otherwise, CE cultures were incubated with 3 ml of Eagle's medium containing actinomycin D (5 μ g/ml) for 2

hr at 37 C. The cultures were then incubated for 1 hr at 37 C with 0.5 ml of a suspension of WEE virus (10 plaque-forming units per cell). The cultures were washed three times with phosphate-buffered saline, given 5 ml of Eagle's medium containing 2% dialyzed calf serum, and incubated at 37 C.

Preparation and analysis of cytoplasmic extracts from CE cells. Monolayer cultures of infected cells were washed three times with ice-cold phosphate-buffered saline. The cells were collected after scraping them from the dishes and centrifuging them at $800 \times g$ for 10 min at 4 C. The cell pellet was suspended in cold solution D [0.01 M tris(hydroxymethyl)aminomethane (Tris), 10^{-3} M KCl, and 10^{-4} M $MgCl_2$, pH 7.4]. The cells were allowed to swell for 8 min at 4 C, at the end of which time they were disrupted by using 10 strokes in a Dounce homogenizer (Kontes Glass Co., Vineland, N.J.). The disrupted-cell suspension was then centrifuged at $10,000 \times g$ for 10 min at 4 C. The supernatant fraction represented the cytoplasmic extract.

Sucrose gradients of 7.5 to 30% (w/w) or 15 to 30% (w/w) in solution D were used to analyze the cytoplasmic extracts. When a Spinco SW25.1 rotor was used, 2 ml of the extract was layered on the gradient; only 1 ml was layered when the SW25.3 rotor was used. The centrifugation was carried out at 4 C in a Spinco centrifuge. Fractions were collected after centrifugation and the trichloroacetic acid-insoluble radioactivity in each fraction was determined as described earlier (7).

Sedimentation of WEE virus on a sucrose gradient. WEE virus contained in the media obtained from infected cultures was sedimented on sucrose gradients. Sucrose gradients of 5 to 35% (w/w) were made in borate saline as described earlier (7). A Spinco SW50 rotor was used; 0.5 ml of the medium was layered on 4.5 ml of a sucrose gradient. Centrifugation was carried out in a Spinco centrifuge at 40,000 rev/min for 30 min at 4 C. Fractions were then collected and the trichloroacetic acid-insoluble radioactivity in each fraction was determined.

Isolation and analysis of RNA. Samples or fractions obtained from the sucrose density gradients after centrifugation were incubated for 15 min at 37 C with sodium dodecyl sulfate (SDS) at a final concentration of 1.0%. Ribosomal RNA (500 μ g) from mouse cells was added to each sample, followed by 2.5 volumes of 95% alcohol. The RNA was allowed to precipitate during 2 hr of storage at -20 C. The precipitate was sedimented by centrifugation at $10,000 \times g$ at 4 C and dissolved in Tris-SDS buffer (0.01 M Tris, 0.1 M NaCl, 0.001 M ethylenediaminetetraacetate, and 0.5% SDS at pH 7.4). Sucrose gradients of 15 to 30% (w/w) in the above buffer were used to analyze the RNA. Samples (1 ml) of the RNA were layered on a 16-ml gradient of sucrose and centrifuged at 24,000 rev/min in a Spinco SW25.3 rotor at 23 C for 14 hr. Fractions were collected; the RNA of each was precipitated with trichloroacetic acid and radioactivity of the RNA was determined.

Electrophoresis of proteins on polyacrylamide gel. The method reported by Summers et al. (9) to analyze poliovirus proteins was used. The proteins were

solubilized with acetic acid and SDS. Polyacrylamide gels (10 cm in length) were used. The procedures for electrophoresis, fractionation of gels, and radioactivity determinations were similar to those described by Summers et al. (9).

RESULTS

Analysis of cytoplasmic extracts. Infected cells were exposed to ^3H -uridine (100 μC per culture) for either 3 min or 120 min at 6 hr after virus was added to the cells. The cytoplasmic extracts from the two groups of cultures were prepared and analyzed (Fig. 1). After a 3-min labeling period, most of the radioactivity in the cytoplasmic extract sedimented slightly slower than ribosomes

FIG. 1. Sucrose density gradient analysis of cells infected with WEE virus and incubated with ^3H -uridine for two different time periods. At 6 hr after virus was added to CE cultures which had been previously incubated with actinomycin D, ^3H -uridine (100 μC /culture) was added per 6×10^6 cells. The cultures were then incubated for 3 min or for 2 hr. The cytoplasmic extract was prepared as described. A 2-ml amount of the cytoplasmic extract was layered on 30 to 7.5% sucrose gradients in solution D and centrifuged at 22,500 rev/min for 3.5 hr in an SW25.1 rotor at 4 C. The cytoplasmic extract from uninfected chick embryo cells was also centrifuged under the above conditions to determine the position of single ribosomes in the sucrose gradient. Fractions were collected and analyzed for optical density and radioactivity. Cytoplasmic extract from cells labeled for 3 min (—); labeled for 2 hr (---); optical density (—). The chick embryo ribosomes were assigned a value of 74S under these conditions.

with a sedimentation coefficient of approximately 65S. However, after incubation with ^3H -uridine for 120 min, the radioactivity was localized in two regions with peaks at 65S and 140S. Centrifugation of cytoplasmic extracts from uninfected cells under the above conditions failed to show any significant radioactivity in these regions of the gradients.

Sensitivity of the 140S and 65S structures to ribonuclease. Ribonuclease was employed to test the hypothesis that the 140S particles were virus-specific polysomes. Cytoplasmic extracts were prepared from cells incubated with ^3H -uridine for 6 hr after infection with the virus. A part of the cytoplasmic extract was incubated with 1 μg of the ribonuclease per ml for 10 min at 0 C. The cytoplasmic extracts (both ribonuclease-incubated and control preparations) were then analyzed on sucrose gradients (Fig. 2). After incubation with ribonuclease, the radioactivity of the 140S and 65S regions was 60% and 20%, respectively, of the control values. Polyribosomes are completely degraded into single ribosomes under these conditions of incubation with ribonuclease. The resistance of the 140S structures to ribonuclease rules out the possibility that they are virus-induced polysomes.

Viral RNA present in the 140S and 65S structures. It has been reported earlier (7) that the virion of WEE virus contains RNA which sediments in sucrose gradients with a coefficient of 40S. Furthermore, infected cells contain two more types of single-stranded viral RNA which sediment at rates of 26S and 15S. Therefore, the type of viral RNA present in the above cytoplasmic structures was of interest. Figure 3 shows the sedimentation profiles of RNA extracted from the 140S and 65S structures which were isolated from cytoplasmic extracts of infected cells. RNA extracted from the 140S structure sedimented with coefficients of 40S and 26S. The 40S type of RNA accounted for about 60% of the total radioactivity in the RNA extracted from the 140S structure. Only the 26S type of RNA was recovered from the 65S structure.

Susceptibility of the RNA in the 140S structure to ribonuclease. Incubation of the cytoplasmic extracts with ribonuclease reduced by 40% the amount of radioactivity which sedimented in the 140S region. Since we knew that the 140S structure contains only single-stranded RNA, the above results suggested that it might be partially susceptible to degradation by the ribonuclease. The extent of the degradation was of interest. Figure 4 shows the sedimentation profiles of RNA isolated from control and ribonuclease-treated 140S particles. The RNA isolated from the

particles incubated with ribonuclease was heterogeneous and sedimented over a region in the sucrose gradient corresponding to RNA of 10S to 4S. Therefore, although incubation of the 140S structure with ribonuclease did not alter its sedimentation rate, the RNA present had been partially degraded. The breakdown of the RNA in the 140S structure was effected with as little as 0.001 μ g of ribonuclease per ml. The presence of residual ribonuclease attached to the 140S

FIG. 2. Resistance of the 140S structure to ribonuclease. Cytoplasmic extract was prepared from cells incubated with actinomycin D, infected with WEE virus, and then labeled with 5 μ C of 3 H-uridine per 6×10^6 cells for 6 hr. The extract was prepared with solution D as described. A sample of the extract was incubated with ribonuclease as described. The untreated cytoplasmic extract and the extract incubated with ribonuclease were analyzed on sucrose gradients prepared in solution D. Centrifugation was for 4 hr at 24,000 rev/min in an SW25.3 rotor at 4°C. Dashed line, ribonuclease; solid line, no ribonuclease.

FIG. 3. Sucrose density gradient analysis of the RNA isolated from the 140S structure and the 65S structure. The cytoplasmic extract from cells infected and labeled with 3 H-uridine for 6 hr following virus addition was fractionated on a sucrose gradient as described in Fig. 1. The fractions representing the 140S and 65S regions were pooled separately; viral RNA was then isolated and analyzed on sucrose density gradients as described. (a) RNA from the 140S structure; (b) RNA from the 65S structure. Arrows indicate the positions where the 28S and 18S ribosomal RNA sediment on similar sucrose density gradients.

particles after sucrose density gradient centrifugation may cause the breakdown of the RNA during the isolation procedure. At present, this possibility cannot be ruled out.

Presence of virus-specific protein in 140S particles. Radioactive amino acids incorporation data indicated that the 140S particles contained protein as well as viral RNA. By analyzing the proteins present in the 140S structure and the mature WEE virus by electrophoresis, proof was obtained that the protein present in the 140S structure was a virus-specific protein. Purified WEE virus labeled with 14 C-amino acids was obtained by methods described earlier (7). The 140S structure was obtained from sucrose density gradient fraction analysis of infected cytoplasmic extracts. Two peaks of radioactivity were seen on polyacrylamide gels when the proteins from WEE virus were analyzed (Fig. 5). The relative mobility of type I protein was only half that of type II, while the amount of radioactivity was threefold higher than that of type II protein. These results are similar to those reported by Strauss et al. (8) and Yin (*personal communica-*

FIG. 4. Sucrose density gradient analysis of viral RNA isolated from the 140S structure after incubation with ribonuclease. The cytoplasmic extract from cells infected and labeled with ^3H -uridine was isolated as described. A sample of the extract was then incubated with ribonuclease at $1\ \mu\text{g/ml}$ for 10 min at 0°C . The untreated and ribonuclease-treated extracts were fractionated on sucrose gradient as in Fig. 1. Peak fractions were pooled; RNA was isolated and then analyzed. Solid line, RNA from untreated 140S structure; dashed line, RNA from 140S structure obtained from cytoplasmic extract incubated with ribonuclease.

tion), who analyzed Sindbis virus. Almost 75% of the radioactivity present in the 140S structure moved in the polyacrylamide gel as type II protein. The apparent presence of some radioactivity in the area of type I protein is probably due to contamination, since the original sample analyzed was obtained by one-step sucrose gradient fractionation. The 140S structure contained at least one of the virus-specific proteins and two types of viral RNA.

Similarity of the 140S structure to the viral core produced by disruption of the virion with deoxycholate. The RNA-containing inner core of Sindbis virus can be released from the virion by incubating the virion with deoxycholate (8). It has been reported that the core produced also possessed a sedimentation rate of 140S on sucrose gradients and contained one of the two structural proteins found in intact virion. The following experiment was therefore performed to test whether the viral core from WEE virus can be prepared by using deoxycholate and whether similarities existed between this viral core and the 140S structure found in infected cells.

Tritiated uridine-labeled WEE virus was prepared and purified by methods described earlier

FIG. 5. Analysis by polyacrylamide gel electrophoresis of the protein present in the 140S structure and in mature WEE virus. ^{14}C -amino acid-labeled WEE virus was prepared and purified as previously described (9). The 140S structure was isolated from cytoplasmic extracts prepared from cells incubated with actinomycin D, infected with WEE virus, and then labeled with ^{14}C -amino acids from the 6th to the 12th hr after virus addition. The gel was 10% polyacrylamide, 10 cm in length. Electrophoresis was carried out at 25°C . Migration was from left (cathode) to right (anode). Solid line, radioactivity from purified virus; dashed line, radioactivity from 140S structure.

(7). A sample of the virus was then incubated with deoxycholate at a concentration of 0.2% at 37°C for 10 min. Samples of the above, as well as of the original virus preparation used, were analyzed on separate sucrose gradients. The profiles of radioactivity found in both sucrose gradients are presented in Fig. 6a. Most of the radioactivity from the deoxycholate-treated virus sedimented with a coefficient of 140S; those present in the intact virus sedimented at about 260S. These results are quite similar to those reported for Sindbis virus (7).

Figure 6b shows the results obtained when ^3H -uridine-labeled virus was incubated with deoxycholate and then was sedimented on a sucrose gradient with a sample of ^{14}C -uridine-labeled 140S structure obtained from cytoplasmic extracts of infected cells. Most of the ^3H -labeled viral core and ^{14}C -labeled 140S structure sedimented at a rate of 140S. The 140S structure apparently underwent some degradation, since there was some slower sedimenting radioactivity due to ^{14}C -uridine. However, these results show that the viral core isolated from WEE virus using deoxycholate was similar in its sedimentation

FIG. 6. Sedimentation of the 140S structure and WEE virus incubated with deoxycholate. (a) Dashed line, ³H-uridine-labeled WEE virus; solid line, ³H-uridine-labeled WEE virus incubated with 0.2% deoxycholate before centrifugation on sucrose gradient. (b) ¹⁴C-uridine-labeled 140S structure was obtained from fractions by centrifugation of ¹⁴C-uridine-labeled cytoplasmic extract as described previously. A sample of such a preparation was mixed with a sample of ³H-uridine-labeled WEE virus incubated with 0.2% deoxycholate as described. The mixture was then centrifuged on a sucrose density gradient. Fractions were collected and radioactivity was determined as described. Solid line, ³H-uridine-labeled WEE virus incubated with deoxycholate; dashed line, ¹⁴C-uridine-labeled 140S structure.

rate to the 140S structure isolated from infected cells. Only the type II viral protein can be detected in the viral core prepared by deoxycholate and, in this respect, it was similar to the 140S structure. Also, it was found that both the viral core and the 140S structure were not infectious for CE cells and, unlike mature virus, possessed no hemagglutinin.

Absence of choline in the 140S structure. It has been reported that lipids constitute about 50% of the dry weight of WEE virus (11). It has also been shown that the lipids present in the host cells serve as the source for the virus-specific lipids (5). Thus, it was of interest to determine whether the 140S structure contained lipids derived from the host cells. Chick embryo cells were incubated with medium containing ¹⁴C-choline for 16 hr to label the cellular lipids. The cultures were then incubated with actinomycin D, as described earlier, and infected with the virus. Medium containing 5 μ Ci of ³H-uridine per ml was added to the cultures 1 hr after addition of virus, and the cultures were then incubated for 6 hr. The cytoplasmic extract was prepared from the cells and the extract was analyzed by sucrose density gradient centrifugation. Figure 7 shows the results of one such experiment. No distinct peak of radioactivity due to ¹⁴C coincided with the peak of radioactivity due to ³H-uridine sedimenting as 140S. The faster sedimenting peak

FIG. 7. Sucrose density gradient analysis of the cytoplasmic extract prepared from cells previously labeled with ¹⁴C-choline before incubation with actinomycin D and then infected with WEE virus. ³H-uridine was used to label the cells from 1 to 6 hr after addition of the virus. Dashed line, radioactivity of ¹⁴C-choline; solid line, radioactivity of ³H-uridine.

of ¹⁴C was found by determination of infectivity to be due to mature virus. Thus, little if any cellular lipid was associated with 140S particles.

The above data provide evidence that the cytoplasm of infected cells contains a viral substructure which is the inner RNA-containing component of mature virus. We therefore assumed that this structure represents some stage in the maturation of the WEE virus. Thus, the 140S structure may function as a precursor to the mature virus. The following experiments were performed to test whether the 140S structure found in infected cells had any role in the development of WEE virus.

Rate of synthesis of 140S structure and mature virus in infected cultures. Several CE cultures were incubated with actinomycin D and then infected with the virus. The cultures were washed three times with phosphate-buffered saline after a 1-hr contact of the virus with the cells at 37 C. Eagle's medium containing 2% dialyzed calf serum was added to the cultures, which were then incubated at 37 C. Replicate cultures were labeled with ³H-uridine (100 μ Ci/culture) at several times after the addition of virus to the cultures. Incorporation was allowed to occur for 4 hr, and then the cultures were harvested. The infected cells and

the medium were separated by centrifugation at $5,000 \times g$. The cell pellet was washed twice with cold Eagle's medium to remove attached virus particles, and the washings were pooled and mixed with the original medium. Labeled virus present in the above medium was assayed by subjecting samples to analysis by sucrose density gradients. Cytoplasmic extracts were prepared from the cell pellets, and the 140S structure present was assayed by sucrose density gradient centrifugation.

In one experiment (Fig. 8), ^3H -uridine was added to the infected cultures from 1 to 4 hr, 4 to 8 hr, 8 to 12 hr, 12 to 16 hr, and 20 to 24 hr after the cells were exposed to the virus. The results are expressed in per cent of the maximal amount of labeled virus or 140S found at various intervals. The rate of incorporation of ^3H -uridine into the 140S structure was found to be the highest in the 4- to 8-hr period, while the rate of the

FIG. 8. Synthesis of 140S structure and virus at various times after infection. Infected cultures were labeled with ^3H -uridine ($100 \mu\text{c}/\text{culture}$) for 4 hr at several times after infection. The medium and the cells were separated by centrifugation. Cytoplasmic extracts were prepared from the samples of the infected cells. Samples of the extracts were then analyzed for the 140S structure on sucrose density gradient as described earlier. The radioactivity contained in the fractions representing the 140S structure was then calculated. Portions of the medium from each sample were analyzed on appropriate sucrose density gradient for free-labeled virus. Radioactivity contained in the fractions representing the mature virus was calculated. Points shown in the figure for each time interval represent the per cent radioactivity based on the maximal incorporation which occurred during the various time intervals after infection.

appearance of the labeled virus was maximal in the 8- to 12-hr period. These values are therefore taken as 100, and the values obtained for the other intervals are expressed as per cent of the maximum. The rate of synthesis of the 140S structure reaches a maximum before the appearance of the virus in the medium. The differential rate of the synthesis of the 140S structure and the labeled virus suggests a precursor product relationship between the 140S structure and the virus particles.

A pulse-chase experiment was performed by using ^3H -leucine. Several cultures were infected with the virus. The cultures were washed three times with phosphate-buffered saline after a 1-hr contact with the virus, and Eagle's medium lacking leucine was added to the cultures which were then incubated. Tritium-labeled leucine ($100 \mu\text{c}/\text{culture}$) was added to the cultures at the 7th hr after the addition of the virus to the cultures. Incorporation was allowed to occur for 1 hr, at the end of which time all the cultures were washed five times with warm phosphate-buffered saline. Then the cultures were incubated with Eagle's medium containing $1 \mu\text{g}$ of leucine per ml. Cultures were harvested at various times after the addition of unlabeled leucine. The medium and the cells were assayed for labeled virus and 140S structure.

In one experiment (Fig. 9), the amount of 140S structure in the infected cells decreased progressively during the chase with unlabeled leucine. The loss in the amount of the label in the cytoplasmic extract can, however, be accounted for in the medium as labeled virus particles. The data presented in Table 1 show the total radioactivity lost from the 140S structure and the radioactivity appearing in extracellular virus at the various intervals during the time of the chase. There is a corresponding increase in the amount of the labeled virus appearing in the medium during the chase period. This apparent transfer of the label from the prelabeled 140S structure to the virus particle suggests that the preformed 140S structure may function as a precursor to the mature virus. Experiments using cycloheximide during the chase period indicate that the transfer of the label from the 140S structure to the virus particle could occur in the absence of new protein synthesis. These results therefore indicate that the 140S structure can function as the precursor to the virus particle. Also, in 15 min after the addition of the unlabeled leucine, labeled virus appears in the medium. This indicates that the conversion of the 140S structure to the virus requires only about 15 min under the above experimental conditions.

FIG. 9. Effect of chasing infected cultures prelabeled with ^3H -leucine with unlabeled leucine. Infected cultures were labeled with ^3H -leucine ($100 \mu\text{c}/\text{culture}$) for 1 hr at the end of the 7th hr after the addition of virus to the cultures. The cultures were washed five times with phosphate-buffered saline at the end of the pulse period; then Eagle's medium containing 1 mg/ml of unlabeled leucine was added to cultures before incubation at 37°C . Cultures were harvested at various times after the addition of the unlabeled leucine. The infected cells were used to prepare the cytoplasmic extract; the medium was used to analyze the amount of labeled virus present. Both the cytoplasmic extracts and the media were analyzed by sucrose density gradient for the 140S structure and virus, respectively.

DISCUSSION

Our data provide evidence that the cytoplasm of the cells infected with WEE virus contains a structure other than the virion. The 140S structure differs from the mature virus in the following respects. (i) It has a lower sedimentation rate than the virion which sediments on sucrose gradient at 260S. (ii) The 140S structure contains, in addition to viral RNA, only one protein compared to the two types of protein found in the virion. This finding is similar to that reported on the nucleocapsid protein released from Sindbis virus with deoxycholate (8). (iii) It is not infectious for CE cells and, unlike mature virus, possesses no hemagglutinin or lipids. Nucleo-

TABLE 1. Appearance of radioactivity in the extracellular virus from infected cultures during a pulse-chase experiment using leucine

Time after chase with unlabeled leucine	Radioactivity present ^a	
	140S structure	Extracellular virus
min		
0	2,900	less than 5
15	2,485	250
30	2,300	520
60	2,060	970

^a Values for the total radioactivity contained as the 140S structure and as the extracellular virus at different intervals during the chase were calculated from the data presented in Fig. 9. Radioactivity contained in the peak fractions representing 140S in the cytoplasmic extract or virus in the medium was summed up for each interval during the chase. Values given above were normalized for the difference in the volume of the cytoplasmic extract and the extracellular medium.

capsids or the cores isolated from WEE virus by incubation with deoxycholate have properties similar to those just described for the 140S structure. The 140S structure described here is identical in many respects with those found in cells infected with SFV (3). Therefore, this structure isolated from the infected cells probably represents the inner RNA-containing core of the virion. Such a view is also borne out by electron microscopic examination of cells infected with WEE and SFV viruses (1, 4). Two steps can be distinguished in the formation of the virus. The first step involves the appearance of virus nucleoid in the cells, and the subsequent step consists of the incorporation of the virus nucleoid into the virus particle by the acquisition of an envelope derived from the cell membrane. Friedman and Berezsky (3) have shown that the 140S structure found in cells infected with SFV has the same morphology under the electron microscope as the viral nucleoid found in thin sections of the infected cell. The 140S structure, as described here, represents the viral nucleoid, since it possesses properties similar to those found for the viral core. We have shown by biochemical means what probably represents the same two steps. The results of the experiment involving a pulse-chase using ^3H -leucine indicate that the loss of the label from the 140S structures in the cytoplasm during the period of chase can be accounted for as free virus found to be released into the medium. This apparent transfer of the label can occur in the absence of any de novo protein synthesis. The prelabeled 140S structure may pick up the

additional viral protein from a pool of the viral protein in the cell and appear in the medium by passage through the cell membrane. The temporal sequence of the formation of the labeled 140S and the labeled virus also agrees with the belief that the 140S structure plays the role of a precursor to the virus particle. It is not known whether the acquisition of the lipoprotein outer membrane occurs in more than one step. It should be possible to isolate subviral structures containing both viral proteins and the RNA if the 140S structure acquires the membrane protein prior to the acquisition of the membrane. So far, however, we have not been able to isolate any such structure. Therefore, it is possible that the acquisition of the membrane protein by the virus nucleoid occurs at the same time that it acquires cell membrane.

Some of the results described here differ from those reported with SFV (3). The 140S structure obtained from WEE virus-infected cells contained both the 40S and 26S types of viral RNA, whereas the similar structure from SFV-infected cells contained only 42S RNA. Both types of RNA are contained also in the 140S structure from cells infected with Sindbis virus (2, 12). The action of ribonuclease on the 140S structure also differed from that reported with SFV. When the cytoplasmic extracts were incubated with ribonuclease, there was always a loss of radioactivity present as ³H-uridine in the 140S structure of WEE virus, and the RNA isolated under these conditions possessed sedimentation coefficients of 10S to 4S. However, ribonuclease did not affect the sedimentation rate of the 140S structure on a sucrose gradient. Our findings indicate that the association of the viral protein and the RNA in the nucleocapsids of WEE virus is not a complete barrier to the enzymatic action of ribonuclease. At present, the significance of these apparent differences between the nucleocapsids of WEE virus and SFV is not clear.

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