Virus-Specific Proteins Synthesized in Cells Infected with *RNA*⁺ Temperature-Sensitive Mutants of Sindbis Virus

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All Sindbis virus temperature-sensitive mutants defective in "late" functions were systematically surveyed by acrylamide-gel electrophoresis for similarities and differences in the intracellular pattern of virus-specific proteins synthesized at the permissive and nonpermissive temperatures. Only cells infected with mutants of complementation group C showed an altered pattern. At the nonpermissive temperature, these mutants failed to induce the synthesis of a polypeptide corresponding to the nucleocapsid protein and instead overproduced a protein of higher molecular weight than either viral structural protein. This defect was shown to be irreversible by the finding that ³H-leucine incorporated at 41.5 C specifically failed to appear in the nucleocapsid of virions subsequently released at 29 C. Attempts to demonstrate a precursor protein in wild-type infections were inconclusive.

Temperature-sensitive (*ts*) mutants of Sindbis virus are unable to grow in cells incubated at 40 C, a condition that is permissive for growth of wild-type Sindbis virus (2). All *ts* mutants fall into one of two major categories: *RNA*⁺ or *RNA*⁻. *RNA*⁻ mutants are defective in viral ribonucleic acid (RNA) synthesis at the nonpermissive temperature. *RNA*⁺ mutants are able to make viral RNA but are defective in some other maturational step at the nonpermissive temperature. Genetic analysis (3) has allowed the subdivision of the *RNA*⁺ mutants into three complementation groups: C, D, and E. Further studies have correlated the genetic, biochemical, and functional defects (4, 5, 12, 21). Members of complementation group C (*ts*-2, *ts*-5, *ts*-13) have a presumed defect in the nucleocapsid protein, as all of these mutants fail to form intracellular nucleocapsids at 40 C. Complementation group D members (*ts*-10, *ts*-23) have a presumed defect in the envelope protein, as cultures infected by either of these mutants at 40 C fail to hemadsorb goose red blood cells. The only member of complementation group E, *ts*-20, has a still unidentified defect.

Recently, Strauss et al. (17) reported that *RNA*⁺ *ts* mutations in the Sindbis virus genome can alter the pattern of intracellular virus-specific proteins revealed by acrylamide-gel electrophoresis of infected monolayers. They found that cells infected with *ts*-13 at 40 C produced a profile of virus-specific proteins that differed

markedly from the wild-type pattern. We have systematically examined all *ts* mutants defective in "late" functions for similarities and differences of virus-specific proteins within each complementation group and among the groups. The results obtained with the mutants of complementation group C led us to suspect that cells infected by wild-type Sindbis virus might contain a precursor protein that was cleaved to produce functional viral proteins of lower molecular weight. Hence, we also explored experimental conditions that seemed likely to demonstrate a precursor-product relationship among the intracellular virus-specific proteins.

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 (13). The isolation and characterization of *ts* mutants of Sindbis virus have also been reported (2-5, 12).

³H-leucine labeling of infected monolayers. Inocula of 10 to 100 plaque-forming units (PFU) per cell were allowed to absorb to cultures for 1 hr at 22 C. Then the infected cells were incubated at the specified temperatures in Eagle's medium containing 1 μg of actinomycin D per ml, 1 μg of leucine per ml, and 3% rabbit serum. When the labeling interval was to be 15 min or less, dialyzed rabbit serum was used. The infected cultures were washed several times with warm Hanks' balanced salt solution (BSS) before radioactive medium was added. Any additional incubation after exposure to ³H-leucine

(Schwarz BioResearch, Inc., Orangeburg, N.Y.) was in fresh medium containing four times the normal quantity of unlabeled leucine.

Temperature-shift experiments. Monolayers infected by *ts* mutants were initially incubated at the permissive temperature (29°C) to permit the infection to become established. To achieve a rapid transition to the nonpermissive temperature (41.5 to 42°C), the culture bottles were completely filled with warm BSS containing 3% rabbit serum and allowed to stand upright for 3 min in a water bath of the appropriate temperature. This fluid was then replaced with medium, and further incubation and labeling were carried out in an incubator or submerged in the water bath.

Treatment with amino acid analogues. The analogues (Calbiochem, Los Angeles, Calif.) used were DL-*p*-fluorophenylalanine, L-canavanine sulfate, L-ethionine, and L-azetidine-2-carboxylic acid. To promote the effects of these analogues, infected monolayers were incubated in medium containing dialyzed rabbit serum and lacking phenylalanine, arginine, and methionine. Proline, for which L-azetidine-2-carboxylic acid is an analogue, is not normally present in our medium. The leucine concentration was also reduced in the usual manner to permit efficient incorporation of ³H-leucine. The infected cells were exposed to the analogues in the absence of serum and exogenously added amino acids for 10 min prior to labeling with ³H-leucine.

Purification of ³H-leucine-labeled virus. Medium (20 ml) containing labeled virus was treated with ammonium sulfate as described by Strauss et al. (17). The precipitate was collected by centrifugation and suspended in 1 ml of water. This entire volume was layered on a 15 to 30% glycerol gradient prepared in 0.15 M NaCl, 0.05 M tris(hydroxymethyl)-aminomethane, pH 7.5, and 0.001 M ethylenediaminetetraacetic acid, and was centrifuged in a Spinco SW 25 rotor at 25,000 rev/min for 2 hr at 4°C. Samples of 25 drops were collected, and the fractions that contained most of the viral radioactivity were pooled and diluted to 10 ml with BSS. Purified virus was obtained from this solution by the sucrose-CsCl method described previously (15) except that the upper, linear gradient was constructed with 10 and 20% (w/w) sucrose in phosphate-buffered saline lacking calcium and magnesium (9).

Viral reference proteins. Virus uniformly labeled with ¹⁴C-reconstituted protein hydrolysate was prepared as previously described (15). This material was added to all samples analyzed by acrylamide-gel electrophoresis and served as the standard ¹⁴C-marker for the viral envelope (E) and nucleocapsid (N) proteins (17, 18).

Polyacrylamide-gel electrophoresis. The monolayers and purified virus were prepared for electrophoresis in sodium dodecyl sulfate (SDS) as described previously (15), except that the mercaptoethanol concentration in the dialysis step was raised to one per cent. The increased mercaptoethanol has been reported to decrease aggregation of protein (17). An 18-cm column of gel was divided into 0.05-ml fractions by extrusion as described previously.

Hemagglutination. The capacity of Sindbis virus to agglutinate goose red blood cells was determined by a modification (14) of the technique of Clarke and Casals (7).

RESULTS

Synthesis of virus-specific proteins in cells infected by *ts* mutants of complementation groups C, D, and E. Strauss et al. (17) reported that 3 hr after Sindbis virus infection host protein synthesis in the presence of actinomycin D is inhibited by 90%, and most of the protein synthesis thereafter is virus-specific. Thus, the major radioactive peaks seen on electrophoretic analysis of cells labeled late in the course of infection can be used to characterize the polypeptide products of the viral genome. We incubated infected cells in three distinct ways to detect variations in the electropherograms attributable to changes in temperature. One culture was maintained at 29°C throughout the infection and labeled with ³H-leucine from 6.5 to 8 hr to assure that the synthesis of virus-specific proteins was normal at this permissive temperature. The second culture was incubated at 29°C for 5 hr after infection, shifted to 41.5°C, and labeled from 6 to 7 hr. To establish whether any alterations in the electrophoretic pattern of virus-specific proteins synthesized during the 1-hr pulse at 41.5°C were reversible, a third culture, labeled in the same manner as the second, was returned to 29°C for an additional 1 hr of incubation in nonradioactive medium containing an excess of unlabeled leucine.

The HR strain of Sindbis virus from which the *ts* mutants were derived served as the control. As can be seen in Fig. 1, HR-infected cells gave the same pattern of virus-specific proteins regardless of the conditions of incubation. The monolayers incubated at 29°C for 5 hr and then shifted to 41.5°C for an additional hour before labeling (Fig. 1A) were identical to those maintained continuously at 29°C (Fig. 1C). This is the expected result because HR or wild-type Sindbis virus grows normally at both temperatures.

Mutant *ts*-20, the only member of complementation group E, forms identifiable nucleocapsids and induces hemadsorption at the nonpermissive temperature (5). Thus, the nature of its defective "late" function is unknown. Electrophoretic analysis of the virus-specific proteins found in cells infected by *ts*-20 gave no clue as to its defect. Figure 2 shows that this mutant resembled HR in its synthesis of virus-determined polypeptides under all conditions tested.

Cells infected by mutants of complementation

FIG. 1. Effect of temperature on the synthesis of virus-specific proteins in cultures infected with HR virus. Three cultures with 10 PFU adsorbed per cell were incubated at 29°C in medium containing 1 μ g of leucine per ml and 1 μ g of actinomycin D per ml. After 5 hr, two cultures were rapidly warmed and incubated in fresh medium at 41.5°C while the third remained at 29°C. This third culture (C) was labeled with 3 H-leucine (25 μ Ci/ml; 40 c/mole) for 1.5 hr beginning 6.5 hr after infection and then was prepared for electrophoretic analysis. The two cultures at 41.5°C were labeled for 1 hr beginning 6 hr after infection. At the end of the labeling period, one of these cultures (A) was immediately prepared for electrophoretic analysis; the other (B) was returned to 29°C for an additional hour of incubation in the presence of excess leucine before analysis. Samples of 130 μ liters were resolved by electrophoresis at 5 v/cm for 15 hr at 22°C. The nucleocapsid (N) and envelope (E) proteins were identified by the 14 C marker (O). The polypeptides migrated from left to right. (A) Infected cells shifted from 29 to 41.5°C at 5 hr after infection and labeled from 6 to 7 hr. (B) Infected cells treated as in A, but returned to 29°C from 7 to 8 hr for incubation in unlabeled medium with excess leucine. (C) Infected cells maintained at 29°C and labeled from 6.5 to 8 hr.

group D at the nonpermissive temperatures are unable to synthesize functional viral envelope protein (5, 21). Because both members of this group showed the same pattern of virus-specific

FIG. 2. Effect of temperature on the synthesis of virus-specific proteins in cultures infected with *ts-20*, a member of complementation group E. The procedure used for each of the cultures (A, B, and C) was identical to that described in the legend of Fig. 1.

proteins, only the results obtained with *ts-10* are presented in Fig. 3. As with complementation group E, the profile of virus-specific proteins synthesized by the group D mutants resembled the pattern seen in cells infected with HR.

In contrast, the mutants of complementation group C, known to be unable to form intracellular nucleocapsids at the nonpermissive temperature (5, 21), showed an obvious temperature-sensitive alteration in the electrophoretic pattern of virus-specific proteins synthesized at 41.5°C. We present the electrophoretograms of all three mutants of complementation group C to demonstrate that they are strikingly similar even though each of these mutants was isolated after treatment with a different mutagen (2). The cultures labeled at the nonpermissive temperature (A in Fig. 4-6) showed a marked deficiency in nucleocapsid protein and a discernible decrease in envelope protein. These deficiencies were approximately balanced by an increase in labeled protein with an electrophoretic mobility just less than one-half that of

FIG. 3. Effect of temperature on the synthesis of virus-specific proteins in cultures infected with *ts-10*, a member of complementation group D. The procedure used for each of the cultures (A, B, and C) was identical to that described in the legend of Fig. 1.

the envelope protein marker. With this defective pattern in mind, it can be seen that even the pattern of cells infected at the permissive temperature (C in Fig. 4-6) showed a hint of the change in pattern, for each had increased incorporation into this slowly moving peak although the nucleocapsid and envelope proteins were well defined. The altered pattern of viral protein synthesis appeared to be irreversible, for no change occurred when cells labeled at the nonpermissive temperature were further incubated at the permissive temperature (B in Fig. 4-6).

Incorporation of proteins synthesized at 42 C into virus released at 29 C. A more stringent test for the renaturation of defective proteins labeled at the nonpermissive temperature by cells infected with *ts* mutants would be incorporation of these proteins into virions assembled during subsequent incubation at the permissive temperature. To test for reversible denaturation, cultures infected with HR, *ts-2*, or *ts-10* and incubated at 29 C for 5.5 hr were shifted to 42 C for 2 min

and then labeled with ^3H -leucine for 8 min at this nonpermissive temperature. Further incorporation of label was halted by incubation at 42 C in the presence of nonradioactive leucine (100 $\mu\text{g}/\text{ml}$) for 5 min. Then incubation at 29 C was resumed for 2.5 hr to allow assembly of functional labeled structural protein into virions (15). This short exposure to the nonpermissive temperature was required because virus production was relatively poor in cells maintained for longer times at the high temperature. The virus released during the final incubation at 29 C was purified as described in Materials and Methods, and both the monolayers and the purified virus were analyzed by acrylamide-gel electrophoresis.

The electrophoretic patterns of the virus-specific proteins in the extracts of infected cells labeled under these new conditions were very similar to those shown in Fig. 1A, 3A, and 4A, and are therefore not presented here. Figure 7 shows incorporation of proteins synthesized at 42 C into virions released during incubation at 29 C. A comparison with HR showed that very little labeled nucleocapsid protein found its way into the virions of *ts-2*, proving that its defect was indeed irreversible. In contrast, the electrophoretic analysis revealed no difference between *ts-10* and HR virus. This suggested that the temperature-sensitive defect in the envelope protein of mutant *ts-10* was reversible.

Yin (20) described a reversible defect in the envelope protein of the other member of complementation group D, *ts-23*. She found that *ts-23* failed to exhibit hemagglutination at 37 C although this assay was normal at 25 C. Prolonged incubation at 37 C, however, did not eliminate the ability of the virus to hemagglutinate at 25 C, and thus the defective behavior was completely reversible. We examined *ts-10* with a similar test and included mutant *ts-23* and wild-type virus for comparison. Our results confirmed the observations of Yin, but mutant *ts-10*, in contrast to *ts-23*, hemagglutinated normally at 37 C. We therefore repeated the experiment at 40 C, the maximal temperature for hemagglutination by the wild-type virus. Even this temperature (Table 1) failed preferentially to reduce the hemagglutinin titer of mutant *ts-10*. Thus, we were unable to find supporting evidence for the reversibility of the defect in *ts-10*. We shall return to this question in the Discussion.

Role of potential precursor protein evident in cells infected by group C mutants at 41.5 C. We have shown that cells infected by any member of complementation group C at 41.5 C accumulated excessive amounts of a relatively high molecular weight protein. Concurrently, the

FIG. 4-6. Effect of temperature on the synthesis of virus-specific proteins in cultures infected with members of complementation group C: *ts-2* (Fig. 4), *ts-5* (Fig. 5), and *ts-13* (Fig. 6). The procedure was identical to that described in the legend of Fig. 1. (A) infected cells shifted from 29 to 41.5 C at 5 hr after infection and labeled from 6 to 7 hr. (B) Infected cells treated as in A, but returned to 29 C from 7 to 8 hr for incubation in unlabeled medium with excess leucine. (C) Infected cells maintained at 29 C and labeled from 6.5 to 8 hr.

synthesis of nucleocapsid protein was drastically reduced. Both of these effects could be explained if the overproduced protein were a precursor of the nucleocapsid protein and this precursor could not be cleaved by the normal mechanism at the nonpermissive temperature. To determine whether the protein accumulated by group C mutants was indeed a true precursor, we examined cultures infected by wild-type virus under conditions most likely to reveal such a molecule.

The length of the labeling interval is an important factor in the pattern of virus-specific proteins revealed by acrylamide-gel electrophoresis of infected monolayers. In poliovirus-infected cells (11, 19), a large polypeptide, NCV P 1, was poorly labeled when the cytoplasm was examined after labeling for more than 30 min or after a pulse of radioactivity followed by a long chase. Conversely, NCV P 1 was highly labeled if the cells were harvested within about 15 min after addition of radioactive amino acids or after a pulse of radioactivity followed by a short chase. Thus, we first studied Sindbis virus-infected cultures after exposure to ^3H -leucine for progressively shorter intervals of time. Replicate cultures infected with wild-type virus for 4.5 hr at 37 C were labeled with ^3H -leucine and then immediately prepared for electrophoretic analysis.

Figure 8 demonstrates that the major virus-specific polypeptides normally observed after long-term labeling were all also labeled with short pulses. The electrophoretic pattern seen after 2 min of exposure to radioactive leucine

did not differ significantly from the 1-hr labeling pattern shown in Fig. 1. Moreover, as the labeling time was progressively reduced to 1 min and to 30 sec, there was no marked preferential labeling of the supposed precursor protein, and the lower molecular weight structural proteins of the virion remained well defined. Although these data lend no support to a precursor role for the polypeptide with just less than one-half the electrophoretic mobility of the envelope protein, we did observe a change in the distribution of the protein migrating only two fractions behind the envelope protein. The presumed conversion of this protein to envelope protein has also been noted by Strauss et al. (17).

Our second approach to establishing a precursor-product relationship was to treat infected cultures with various amino acid analogues—fluorophenylalanine, canavanine sulfate, azetidine-2-carboxylic acid, and ethionine. This technique has been successfully employed in poliovirus infections to detect a previously unsuspected high molecular weight protein (11). Monolayers infected with wild-type virus for 4.25 hr were exposed to the analogues in the absence of serum and amino acids for 10 min prior to the addition of ^3H -leucine. As can be seen in Fig. 9, the analogues significantly reduced incorporation of ^3H -leucine during the 15-min pulse. However, the control and analogue-treated cultures showed a similar pattern of virus-specific protein synthesis. No new peaks were evident, and the distribution of label among

FIG. 7. Incorporation into virions of structural proteins synthesized at the nonpermissive temperatures. Cultures in 32-oz bottles were infected with *ts-2*, *ts-10*, or *HR* virus as described in the legend of Fig. 1. After 5.5 hr, the monolayers were washed and incubated in fresh, prewarmed medium at 41.5 C for 2 minutes. The cells were then labeled with ^3H -leucine (300 $\mu\text{C}/\text{ml}$, 58 c/mmole) for 8 min at 41.5 C. After an additional 5 min of incubation at 41.5 C in the presence of excess leucine, the cultures were returned to 29 C for 2.5 hr. The virus released during the incubation at 29 C was purified and prepared for electrophoretic analysis as described in Materials and Methods. Samples of 125 μl were resolved by electrophoresis at 5 v/cm for 22.5 hr at 22 C. The nucleocapsid (N) and envelope (E) proteins were identified by the ^{14}C marker (O).

TABLE 1. Hemagglutinin titers at 22 and 40 C

Virus	Temp of hemagglutination assay	
	22 C	40 C
<i>ts-10</i>	1,024	512
<i>ts-23</i>	512	<2
Wild type.....	512	128

the various polypeptides was approximately the same in the two cultures. A slight increase in the relative amount of the supposed precursor protein was apparent in the analogue-treated cultures, but this was a minor effect. It is clear that

FIG. 8. Pulse labeling of cells infected with wild-type Sindbis virus. Replicate cultures with 100 PFU adsorbed per cell were incubated at 37 C in medium containing 1 μg of leucine per ml, 1 μg of actinomycin D per ml, and 3% dialyzed rabbit serum. After 4.5 hr, the monolayers were washed twice with BSS and pulse-labeled with ^3H -leucine (150 $\mu\text{C}/\text{ml}$, 40 c/mmole) in the absence of serum and unlabeled leucine. After 0.5, 1, or 2 min, the cells were immediately prepared for electrophoretic analysis as described in Materials and Methods.

the analogues did not prevent the appearance of the major structural proteins of Sindbis virus. Thus, this method also failed to provide convincing evidence for a high molecular weight precursor protein in cells infected by wild-type Sindbis virus.

DISCUSSION

The mutants code for an altered protein that assumes a functional configuration when synthesized at the permissive temperature but fails to assume a functional configuration when synthesized at the nonpermissive temperature. Only a missense mutation in the viral genome can explain this behavior, as the protein-synthesizing mechanism of the host cell probably translates the mutant viral genome into the same amino acid sequence at both the permissive and non-

FIG. 9. Effect of amino acid analogues on the virus-specific proteins synthesized in cultures infected with wild-type Sindbis virus. Replicate cultures were infected and incubated as described in the legend of Fig. 8 except that leucine, arginine, methionine, and phenylalanine were omitted from the medium. At 4.25 hr, the cells were washed twice with warm BSS before treatment of one group for 10 min with 2.5 mM DL-p-fluorophenylalanine, 3.5 mM L-canavanine sulfate, 1.8 mM L-ethionine, and 5.4 mM L-azetidine-2-carboxylic acid in the absence of serum and amino acids. At the end of this time, both the experimental and control cells were labeled with ^3H -leucine (50 $\mu\text{C}/\text{ml}$, 40 c/mmole) for 15 min in the presence or absence of analogues, respectively.

permissive temperatures. This is particularly likely in view of the fact that the normal body temperature of the chicken is nonpermissive for our mutants. A missense mutation in the viral genome would in translation produce an altered protein differing from the wild-type in amino acid sequence but not in molecular weight. Thus, the mutant and wild-type proteins should have the same mobility when analyzed by acrylamide-gel electrophoresis in the presence of SDS, a procedure that separates proteins on the basis of molecular weight (16). This was, indeed, the case with the virus-specific proteins synthesized in cells infected with both members of

complementation group D at the nonpermissive temperature. Two physiological tests (5, 20, 21) have shown that these mutants have a defective envelope protein, but this protein had a normal electrophoretic mobility even when synthesized at the nonpermissive temperature.

However, all three members of complementation group C, already known to be defective in making nucleocapsids (5, 21), almost totally failed to induce the synthesis at the nonpermissive temperature of a polypeptide product corresponding to the nucleocapsid protein. At the same time, these three mutants overproduced a polypeptide of a molecular weight higher than either principal structural protein of the virion. A simple hypothesis to link these two observations would be that the overproduced polypeptide is a precursor of the nucleocapsid protein. This supposed precursor accumulated because the process of cleavage was temperature-sensitive. (As used here, cleavage refers to the splitting of a pre-existing peptide bond.)

Jacobson and Baltimore (11) suggested that animal cells cannot interrupt the translation of polycistronic messenger RNA to release the polypeptide products of individual cistrons as bacteria do. They presented evidence that the initial product when poliovirus RNA serves as messenger is a large polypeptide that is subsequently cleaved to form functional viral proteins. We employed both tests used by Jacobson and Baltimore (11) to demonstrate the accumulation of a high molecular weight precursor protein in cells infected by Sindbis virus. Neither very short ^3H -leucine pulses nor the incorporation of amino acid analogues markedly increased the wild-type accumulation of the presumed precursor protein that was so extensively overproduced in cells infected by mutants of complementation group C at the nonpermissive temperature. Similar results were obtained by Friedman (10) using the wild type of a related arbovirus, Semliki Forest virus. But both tests for a precursor are conclusive only if a positive result is obtained. If a large polypeptide were cleaved as it was being synthesized by polyribosomes, no pulse label, however short, could detect its precursor role.

The failure of the amino acid analogues to demonstrate a precursor can also be explained. The mechanism of action of these substances is uncertain. They are probably incorporated and, in the case of poliovirus-determined polypeptides, either prevent recognition of the cleavage site or inhibit the cleavage mechanism. In a presumed arbovirus precursor, the amino acid sequence at the cleavage site may be different from that of poliovirus-specific proteins and

hence less susceptible to alteration by the incorporated analogues. It should be noted, however, that we did observe a slight increase in the supposed precursor peak in infected cells treated with analogues.

Thus, the most likely interpretation of our data is that the polypeptide overproduced in cells infected with group C mutants at the nonpermissive temperature is a precursor protein. Two alternate hypotheses should be considered. First, the precursor protein may simply be a nonspecific aggregate of two or more virus-determined proteins. However, aggregation is unlikely in SDS-mercaptoethanol (17), and we are thus inclined to discount this explanation.

A second, alternate hypothesis is that the Sindbis viral genome may determine a mechanism for the interrupted translation of polycistronic messenger RNA like that seen in bacteria (1, 6). The mutants of complementation group C would then have a temperature-sensitive defect in this mechanism and would thus accumulate precursor proteins at the nonpermissive temperature. A difficulty with this hypothesis is that it predicts a larger precursor protein than was actually found. Using the estimated molecular weights previously assigned to the nucleocapsid (30,000) and envelope (53,000) proteins (17), we calculate (16) the molecular weight of the polypeptide overproduced by mutants of complementation group C to be about 90,000. The possibility remains, of course, that more than one polypeptide of this approximate molecular weight was present in this region of the acrylamide-gel. Since the 42S virion RNA, with a molecular weight of 4×10^6 to 5×10^6 (17), can code for 5×10^5 to 6×10^5 daltons of protein, it is clear that the polypeptide product of the entire viral genome has not been identified. Even if the virion RNA were composed of two segments with approximately one-half this molecular weight (the 26S RNA), as recently suggested by Dobos and Faulkner (8), a larger protein would have been expected. Thus, it is unlikely that a temperature-sensitive defect in the interrupted translation of viral messenger RNA can explain our results. The most likely hypothesis remains that the structural proteins of Sindbis virus are produced by cleavage of high molecular weight precursors and that this cleavage is so rapid as to be undetectable in normal infections. Only when the normal process of cleavage was disturbed by a temperature-sensitive mutation was it possible to demonstrate the existence of a precursor.

If the precursor hypothesis is accepted, we can make certain deductions as to the nature of the defect in the mutants of complementation

group C. When a precursor accumulates at the nonpermissive temperature, either its conformation or the activity of the cleavage mechanism must be temperature-sensitive. After infected cells were shifted to a permissive temperature, an active cleavage mechanism must have been present even if it were determined by the viral genome. But the presumed precursor which accumulated at the nonpermissive temperature did not disappear when the cells were further incubated at the permissive temperature. Thus, the supposed precursor appeared to be irreversibly in a conformation that prevented normal cleavage.

The exclusion of the nucleocapsid protein synthesized at the nonpermissive temperature from *ts*-2 virions subsequently released at the permissive temperature demonstrated the irreversibility of the defect in group C mutants. Here, the results simply confirmed the already known defect in the nucleocapsid protein. However, a similar method would be useful in the identification of the altered structural protein in *ts* mutants of more complex virions where direct functional tests are difficult.

The same experiment with *ts*-10 indicated that its altered envelope protein synthesized at the nonpermissive temperature was incorporated into virions subsequently released at the permissive temperature. This result suggested that the conformation of the envelope protein of *ts*-10 might be reversible. The envelope protein of another member of complementation group D (*ts*-23) has been shown to be reversibly inactivated in hemagglutination tests at various temperatures (20). We were unable to demonstrate this degree of reversibility with the virions of *ts*-10, but such a test does not exclude reversibility in the intracellular conformation of the envelope protein.

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