Thermosensitive Block of the Sabin Strain of Poliovirus Type I

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The thermosensitive defect of the Sabin LSc2ab strain of poliovirus type I was studied. Transfer of infected KB cells from 36 to 38.5 C resulted in 30% inhibition of viral RNA replication but in 90% inhibition of formation of virions. Neither 74S procapsids nor 14S particles were detected in the cells transferred to the nonpermissive temperature. However, procapsids, once accumulated at 36 C, were normally stable at 38.5 C and could transform into virions at that temperature. Viral proteins synthesized at the nonpermissive temperature were not different from those synthesized at permissive temperature, as judged from their pattern in polyacrylamide gel electrophoresis and from the fact that they normally matured into virions when the infected cells were brought back to permissive temperature, even under conditions of inhibition of protein synthesis. This leads to the conclusion that the defect in the Sabin strain studied lies in the assembly of its viral capsid proteins into capsomeres.

The effect of supraoptimal temperatures on the multiplication of poliovirus has been well documented (for a review, see reference 16). Its mode of action, however, remains to be fully elucidated.

Poliovirus ribonucleic acid (RNA) replication is known to be arrested at fairly high temperatures in vivo (8, 23), but this is true for thermoresistant as well as for thermosensitive strains. Moreover, viral RNA polymerase prepared from cells infected with a thermosensitive poliovirus strain is not thermosensitive (19). Therefore, inhibition of virus growth by supraoptimal temperatures does not seem to bear directly on the process of RNA replication, in the case, at least, of the various poliovirus strains which have been studied so far.

We have demonstrated previously that upon a shift of poliovirus-infected cells from 36 to 39.5 C, newly made viral RNA was progressively degraded in the infected cell (8). Such a process occurred regardless of the strain of poliovirus tested, whether thermosensitive or not, which suggested that it was cell mediated. In agreement with this hypothesis, we could demonstrate, since then, that degradation of viral RNA is paralleled by a partial release, from particulate to soluble form, of acid ribonuclease, β-glucuronidase, and acid phosphatase, all enzymes which normally are clustered inside cell lysosomes. We could also demonstrate that degradation of viral RNA is independent of the nature of the cell which is infected and that it is several times ac-celerated in cells whose lysosomes have been rendered fragile by addition of 1% sucrose to the growth medium (R. Wattiaux, Thèse, d'Agrégation, Univ. de Louvain, 1966) (unpublished results). Inhibition of poliovirus RNA replication at elevated temperatures therefore appears to be due primarily to a cellular response involving the release of lysosomal enzymes, which can mask the eventual thermosensitivity of the viral strain studied.

To escape this phenomenon and to study the genetic defect of the Sabin strain of poliovirus type I, we chose to study its replication at temperatures lower than 39.5 C, hoping that, in such conditions, release of lysosomal ribonuclease and subsequent degradation of virus RNA might be kept minimal. Indeed, we found that at 38.5 C, which is the rt (rt is defined as the temperature at which infectious virus yield is inhibited by 90% [15]) of the strain, production of virions was inhibited by 90%, whereas virus RNA replication continued. The specific thermosensitivity of the strain was found to lie in the assembly of its capsid proteins into the various particulate precursors of the virion.

MATERIALS AND METHODS

Viral infection. The virus used was the Sabin LSc2ab strain of poliovirus type I, already passaged for many years in the laboratory. KB cells in Eagle medium were seeded 24 hr before infection at 1.3 to 1.5 × 10⁶ cells per 6-cm plastic petri dish. They were

washed with phosphate-buffered saline (PBS) the following day and infected with 0.2 ml of a virus suspension. The input multiplicity of infection was 50 to 100 plaque-forming units (PFU) per cell. After 45 min of adsorption, fresh medium without serum and supplemented with 3 μg of actinomycin D per ml was added, and the plates were incubated at the indicated temperatures.

RNA synthesis. Virus RNA synthesis was determined by the incorporation of tritiated uridine (0.5 μCi/ml, 25 Ci/mmole) into trichloroacetic acid-precipitable material. For each determination, the cells of a single petri dish were scraped from the plate, cold 10% trichloroacetic acid was added, and the precipitate was centrifuged. The pellet was washed three times with cold 5% trichloroacetic acid and resuspended in 0.5 ml of concentrated NH_4OH, and radioactivity was determined in 10 ml of Bray solution (4).

Cell fractionation and sucrose gradient analysis of cytoplasmic extracts. Cytoplasmic extracts were either prepared by Dounce homogenization in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.4), 0.01 M NaCl, 0.0015 M $MgCl_2$ (2), or by the use of 0.5% Nonidet P-40 in a 0.01 M Tris-hydrochloride (pH 8.5), 0.134 M NaCl, 0.01 M $MgCl_2$ buffer (3). Nuclei were sedimented by centrifugation at 800 × g for 3.5 min.

Analysis of the virions was performed by centrifugation of cytoplasmic extracts for 3 hr at 25,000 rev/min, 3 C, in the SW25.1 Spinco rotor, through 28-ml gradients from 5 to either 30 or 40% sucrose. The gradients were made either in 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M NaCl, 0.0015 M $MgCl_2$ (RSB), or in the same buffer with Mg^{2+} replaced by 0.02 M ethylenediaminetetraacetic acid (EDTA) (NEB), as indicated in the legends to the figures. After centrifugation, the gradients were monitored for optical density at 260 nm, and the acid-precipitable radioactivity in each fraction was determined as previously described (8).

Polyacrylamide gel electrophoresis. The procedure described by Summers, Maizel, and Darnell (22) was followed. Cytoplasmic extracts were incubated for 1 hr at 37 C in the presence of 1/10 volume of glacial acetic acid, 1% sodium dodecyl sulfate (SDS), and 0.5 M urea, and then extensively dialyzed against the electrophoresis buffer (0.5 M urea, 0.1% SDS, 0.1 M sodium phosphate buffer, pH 7.1) containing 0.1% β-mercaptoethanol. Gels, 8 cm long and 6 mm in diameter, were made with 7.5% acrylamide and 0.2% bis-acrylamide and cast in Plexiglas tubing. Electrophoresis was for 6 hr at 10 ma per gel.

RESULTS

KB cells seeded in petri dishes were infected with the Sabin LSc2ab strain of poliovirus and incubated at 36 C for 2.75 hr, after which part of the plates was transferred to 38.5 C. Viral RNA synthesis and intracytoplasmic accumulation of virions were followed at both temperatures as described under Materials and Methods. Upon transfer to the higher temperature, RNA replica-

tion continued for more than 2 hr at a rate which was 70% of that at 36 C and ceased at approximately the same time as in the control culture (Fig. 1A). In contrast, the amount of virions made at 5 hr postinfection at 38.5 C was less than 20% of that made at 36 C (Fig. 1B). This result shows that the inhibition of production of virions at 38.5 C is not due to impaired RNA synthesis but might be due to lack of encapsidation of the RNA at the elevated temperature.

Since it is known that the direct precursor to the virion is the 74S procapsid (12), a block in the assembly of viral RNA and proteins could have resulted in the accumulation of procapsids inside the infected cell. To test this possibility, poliovirus-infected KB cells were labeled for 2 hr with radioactive amino acids at either 36 or 38.5 C. Cytoplasmic extracts were then prepared and analyzed by sucrose gradient centrifugation for the presence of procapsids. Fig. 2A shows that no label was found at the position of procapsids in the extracts from the cells incubated at 38.5 C (open circles). Examination of the material sedimenting more slowly than 74S was also performed, to determine whether 14S subunits which are the precursors to the 74S procapsids (17, 18) were formed at the nonpermissive temperature. No peak of radioactivity was found, however, in the 14S region at 38.5 C (Fig. 2B). This shows that no precursors of the virion can be found in cells incubated at such nonpermissive temperature.

It was questioned next whether these results were due to thermolability of the precursors at high temperature. It is known that, in the presence of 3 mM guanidine, which inhibits poliovirus RNA replication (1), viral procapsids accumulate in the cytoplasm of infected cells and can eventually be converted into virions when the inhibitor is withdrawn (12). To determine whether the procapsids of the Sabin strain of poliovirus, once formed at 36 C, were stable at 38.5 C, infected KB cells were labeled at 36 C in the presence of guanidine, then transferred to 38.5 C in the continuous presence of the inhibitor.

Labeling of the cells was performed in suspension, since it has repeatedly been observed that uptake of labeled amino acids into acid-precipitable material is increased by three- to fivefold in suspended cells as compared to cells maintained in monolayer cultures. KB cells, infected 2.50 hr previously, were detached from their support through the use of EDTA, and suspended in medium with 1/50 the normal amino acids concentration. A mixture of tritiated amino acids and guanidine was then added. After 1 hr of labeling in the presence of guanidine at 36 C, part of the cell suspension was transferred to 38.5 C in the
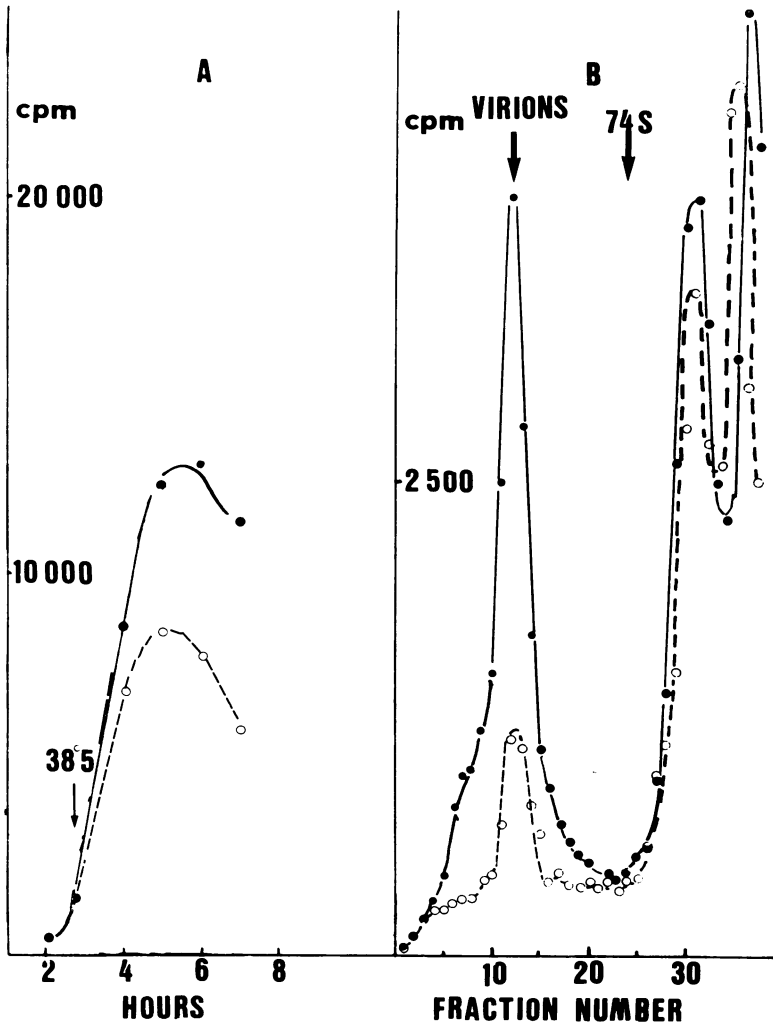
FIG. 1. *Effects of the nonpermissive temperature on RNA synthesis and encapsidation. KB cells seeded at 1.3 ×* 10^6 *cells per petri dish were infected at 36 C with the Sabin strain of poliovirus type I in the presence of 3 μg of actinomycin D per ml. The cells were either kept at 36 C* (●—●) *or transferred to 38.5 C at 2.75 hr after infection* (○---○). *A, Uridine uptake. Tritiated uridine (0.5 μCi/ml) was added at 1 hr after infection, and incorporation of acid-precipitable radioactivity was determined as described under methods. B, Formation of virions. Tritiated uridine (1 μCi/ml) was added at 1 hr after infection. Cytoplasmic extracts were prepared 4 hr later from the cells of five petri dishes. They were then analyzed by centrifugation through 28-ml gradients of 5 to 30% sucrose in NEB for 3 hr at 25,000 rev/min, 4 C, in the SW25.1 Spinco rotor.*

same medium and incubated for an additional 1 hr, whereas another part was kept at 36 C. Cytoplasmic extracts were then prepared and analyzed for the presence of radioactive procapsids. The amount of label in 74S procapsids in the extract from the cells treated with guanidine, labeled for 1 hr at 36 C, and then transferred for an additional 1 hr at 38.5 C (open circles, Fig. 3A) was approximately identical to that found in the cells treated with guanidine and labeled for 1 hr at 36 C (stars, Fig. 3B), and about equal to half

that found in control cells kept for 2 hr in the presence of guanidine at the permissive temperature (closed circles, Fig. 3A). This shows that negligible accumulation of procapsids occurred in the presence of guanidine at 38.5 C, but that procapsids which had previously been formed at 36 C were stable when the cells were further incubated at the nonpermissive temperature.

Procapsids could normally be converted to virions at 38.5 C once guanidine was removed, as shown in the following experiment. A sample from
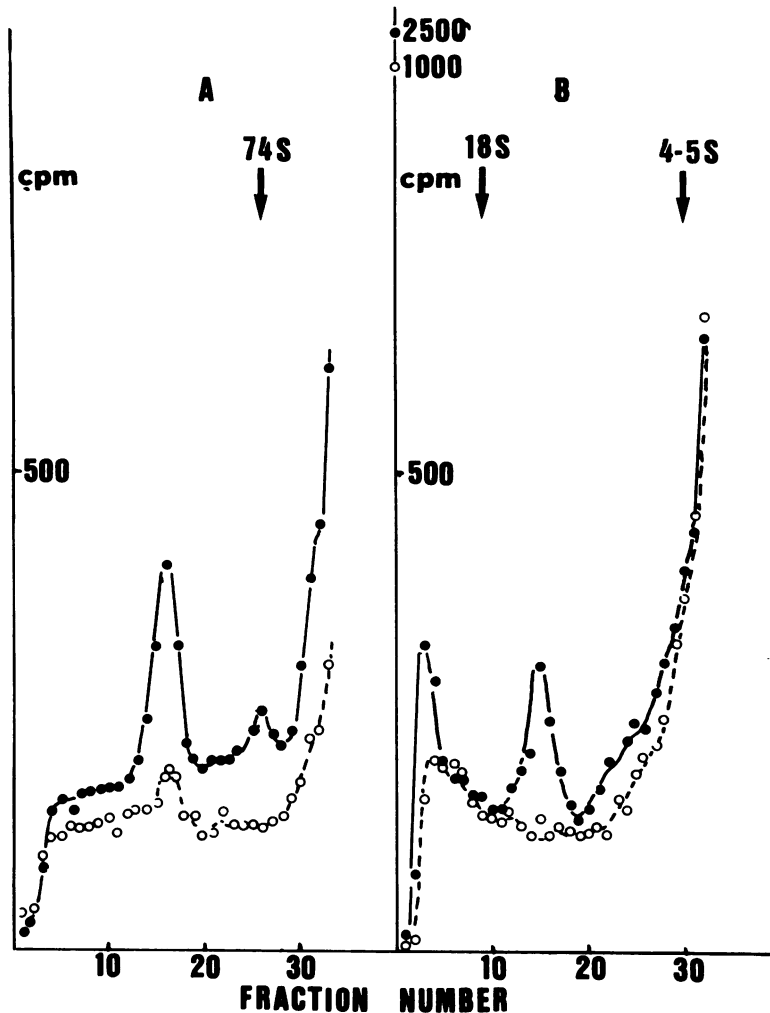
FIG. 2. *Absence of accumulation of procapsids and 14S particles at 38.5 C. Infection was as described in the legend to Fig. 1. At 2.15 hr after infection, the medium was withdrawn, the cell cultures were overlaid with 5 ml of minimal essential medium containing 1/50 the normal amino acids concentration, and a part was transferred to 38.5 C (○--○) whereas another part was kept at 36 C (●—●). Thirty minutes later, both were labeled with tritiated amino acids mixture (5 μCi/ml). Cytoplasmic extracts were prepared from the cells of five petri dishes at 5.5 hr after infection. They were analyzed by sucrose gradient centrifugation through either (A) 15 to 30% sucrose in RSB for 3 hr at 25,000 rev/min, 4 C, in the SW25.1 Spinco rotor or (B) 5 to 20% sucrose, also in RSB, for 40 hr at 25,000 rev/min, 4 C, in the same rotor.*

the cells labeled with amino acids for 1 hr at 36 C in the presence of guanidine as described above was washed twice to remove both label and inhibitor, and the label was chased at either 36 or 38.5 C for 1 hr. Approximately 50% of the labeled procapsids were chased into virions during that time, regardless of the temperature at which the chase was performed (open and closed circles, Fig. 3B). Once formed at 36 C, viral procapsids can therefore mature to virions as well at 38.5 as at 36 C. Furthermore, the same amount of in-

fectious units was recovered under the peak fractions of virions after the chase at either temperature (data not shown).

The preceding experiments indicate that the block responsible for the thermosensitivity of the Sabin strain of poliovirus type I is located very early in the pathway to capsid assembly. The next series of experiments was aimed at determining more precisely which of the early steps was inhibited at high temperature. The first question which was raised was whether the
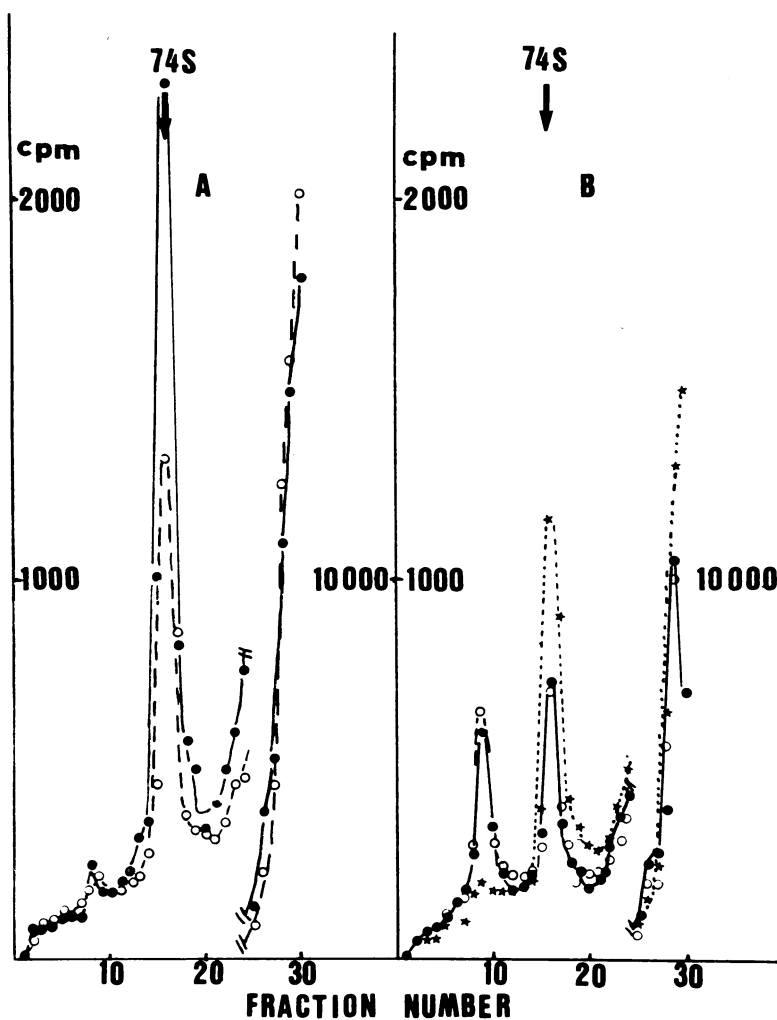
FIG. 3. *Labeling and chase of viral procapsids. At 2.5 hr after infection, infected KB cells were detached with EDTA and suspended in minimum essential medium containing 1/50 the normal concentration of amino acids. The suspension was incubated at 36 C for 15 min, after which 3 mM guanidine and tritiated amino acids mixture (5 µCi/ml) were added. Cytoplasmic extracts were prepared after either 1 or 2 hr of incubation at 36 C (A, ●; B, ★, respectively) or after 1 hr at 36 C plus an additional 1 hr at 38.5 C (○, A). In addition, part of the cell suspension was washed twice after the first hour of incubation at 36 C, to eliminate both guanidine and labeled amino acids. The cells were resuspended in complete medium supplemented with twice the normal concentration of amino acids and further incubated for 1 hr, either at 36 C or at 38.5 C (B, ● and ○, respectively). All cytoplasmic extracts were prepared from 6 × 10⁶ cells. They were analyzed through 36-ml gradients of 5 to 40% sucrose in NEB for 5 hr at 27,000 rev/min, 4 C, in the SW27 Spinco rotor.*

proteins synthesized in the infected cells at 38.5 C were functional, i.e., whether they could be used as such for the eventual encapsidation of viral RNA when the temperature was lowered to 36 C. At 2.75 hr after infection, cells were transferred to 38.5 C, and after 30 min of incubation, labeled with tritiated amino acids for 20 additional min. The label was then chased by excess cold amino acids while the cell culture was transferred

back to the permissive temperature. Cytoplasmic extracts were prepared at various times thereafter and analyzed by sucrose gradient centrifugation for the accumulation of label into virions. In parallel, control-infected cells, kept at all times at 36 C, were labeled and chased in the same manner.

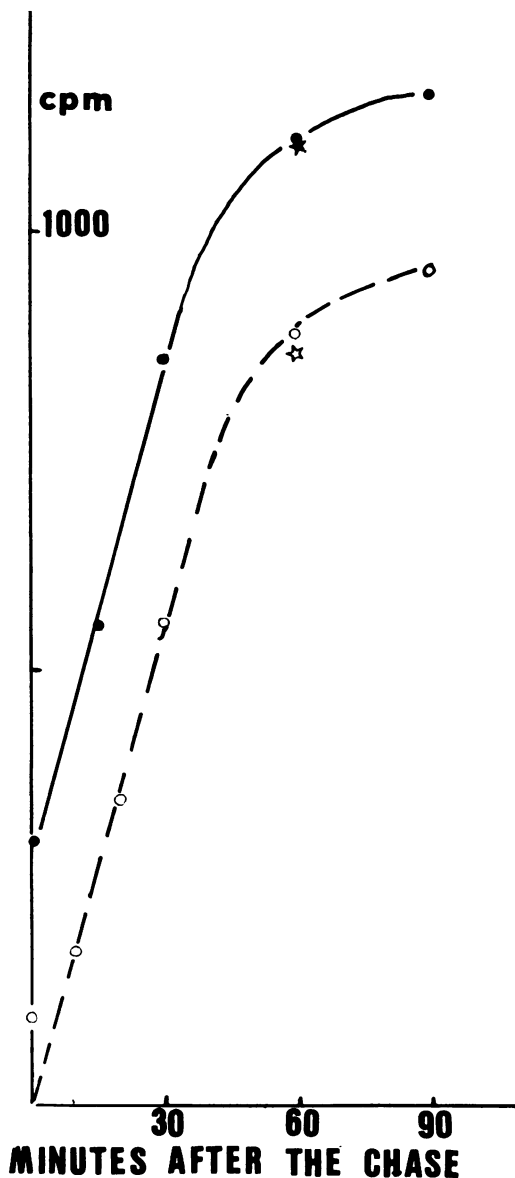The time course of appearance of label in mature virus particles is shown in Fig. 4. In the

FIG. 4. *Formation of virions at 36 C with viral proteins synthesized at 38.5 C. At 2.75 hr after infection, infected KB cells were suspended in minimal essential medium with 1/50 the normal concentration of amino acids. Part of the cell suspension was kept at 36 C (●), whereas another part was transferred to 38.5 C (○). Thirty minutes later, both samples were labeled with tritiated amino acids mixture (5 μCi/ml) for an additional 20 min at 36 and 38.5 C, respectively. The label in both samples was then chased by washing twice and resuspending the cells in complete medium with twice the normal concentration of amino acids and incubating them at 36 C. Cytoplasmic extracts were prepared at various times thereafter and analyzed as in Fig. 1 for accumulation of radioactivity into virions.*

cells labeled and chased at 36 C, the amount of radioactivity accumulating into virions during the chase increased linearly for approximately 40 min and then leveled off to reach a maximum by 90 min after the chase (closed circles). In the cells labeled at 38.5 C and then chased at 36 C (open circles), the accumulation of label into virions followed the same time course as above but the curve was shifted, due to the absence of labeled virions at the time when the cells were transferred to 36 C, and the chase was initiated. Also, the final amount of radioactivity recovered in virions at 90 min after the beginning of the chase was lower. That these results were not due to degradation and reutilization of label was shown by inhibiting protein synthesis through the use of cycloheximide at the time of the chase in both samples of labeled infected cells. No difference in the time-course of the accumulation of label into virions was found when cycloheximide was present during the chase. (For the sake of clarity, only the values corresponding to the samples withdrawn at 60 min after the beginning of the chase have been plotted on Fig. 4 [stars].)

This experiment shows that the inhibition of capsid assembly at nonpermissive temperatures is readily reversible when the temperature is brought back down to normal, and that the proteins which have been synthesized at high temperature are functional, since they can normally assemble into mature particles at 36 C. Moreover, since inhibition of protein synthesis at the time of the chase at 36 C did not prevent the appearance of labeled mature virus particles, it follows that all constituents required for the formation of the viral capsids had been normally synthesized at 38.5 C.

Analysis of the various virus-coded proteins synthesized in the infected cells at both permissive and nonpermissive temperatures was performed through polyacrylamide gel electrophoresis. KB cells were infected for 2.75 hr at 36 C and then half of the culture was transferred to 38.5 C while the other half was kept at 36 C. At 3.25 hr, a tritiated amino acids mixture was added to both samples. Cytoplasmic extracts were prepared after 30 min of labeling and analyzed on 7.5% polyacrylamide gels as described in Materials and Methods. A sample of capsid proteins from purified virions of the Mahoney strain was run in parallel, to provide known molecular weight markers (11, 21). No difference could be detected between the electrophoresis pattern of the

●, *Cells labeled and chased at 36 C;* ○, *cells labeled at 38. 5C and chased at 36 C. In parallel, part of both cultures was chased in the presence of cycloheximide (100 μg/ml) (★ and ☆).*

proteins synthesized in the cells infected with the Sabin strain, whether at 38.5 or at 36 C (compare Fig. 5A and B).

The various peaks detected in the electropherograms were identified by comparing their rate of migration to that of the Mahoney capsid polypeptides. This procedure allowed identification of viral capsid proteins VP_1, VP_2, and VP_3, as shown by the arrows in Fig. 5. It is known that virions of the Sabin strain of poliovirus lack most of VP_1 (5–7). This is reflected also in cytoplasmic extracts, in which VP_1 is barely detectable. The peak to the left of VP_1 is VP_0, since it has the same electrophoretic mobility as that of the procapsid of the Mahoney strain (13, 21), and since it was found in association with VP_3 in the procapsids of the Sabin strain (not shown). Assuming a linear correlation between electrophoretic mobilities and logs of molecular weights, the molecular weights of the two heaviest polypeptides found at the top of the gels in Fig. 5 are

FIG. 5. *Polyacrylamide gel electrophoresis of the proteins synthesized at 36 and 38.5 C. Infected KB cells were labeled with tritiated amino acids mixture (5 μCi/ml) for 30 min at either 36 C (A) or 38.5 C (B), beginning at 3.25 hr after infection. Cytoplasmic extracts were prepared and analyzed on 7.5% polyacrylamide gels as described in Materials and Methods. The arrows refer to the position of the viral proteins from purified Mahoney virions or procapsids analyzed in parallel.*

approximately 1.20×10^5 and 0.9×10^5. These values correspond to those reported by Summers and Maizel (21) for the molecular weights of the noncapsid viral protein NCVP 1 and 2. Although the precise determination of the molecular weights of each of the detected peaks was not attempted, the overall pattern of migration of the various proteins observed here in the cytoplasm of cells infected with the Sabin strain of poliovirus is quite similar to that obtained for the proteins recovered from cells infected with the Mahoney strain, except for the relative absence of VP_1. Also, these results show that there is no detectable difference in the synthesis and cleavage of the viral protein of the Sabin strain, whether the infected cells are incubated at permissive or nonpermissive temperature.

DISCUSSION

These experiments demonstrate that the thermosensitivity of our strain of Sabin poliovirus type I is related to the assembly of its capsid polypeptides. These polypeptides are synthesized at 38.5 C as well as at 36 C, but can only be assembled into procapsids and virions when the temperature is brought back to 36 C. Viral procapsids formed at 36 C are stable, however, at 38.5 C and can mature normally into infective virions at the nonpermissive temperature.

It therefore appears that the defect which characterizes the viral capsid proteins of the Sabin LSc2ab strain is essentially reversible, and involves one of the early steps in assembly, probably at the stage of the formation of capsomeres. Polymerization of the viral protein subunits is prevented at high temperature, perhaps due to a faulty configuration of the polypeptides. It is not known with certainty whether assembly of poliovirus shells is an autocatalytic process or whether it requires the participation of one or several specific enzymes or factors. Although the former mechanism is most likely, in view of the reported in vitro reconstruction of virions of RNA phages (20) or TMV (9) from their RNA and proteins, it has been demonstrated in the case of poliovirus that similar in vitro reconstruction can only occur in the presence of cytoplasmic extract from infected cells (17). Whether this reflects the necessity for a virus-specific enzyme or factor is not known. If this were to be the case, it would provide a basis for understanding the thermosensitivity of the Sabin strain, since thermosensitivity of the factor(s) would preclude the assembly of viral capsid proteins at high temperature, without affecting the stability of already formed particulate structures. If, on the other hand, formation of virions is a self-assembly
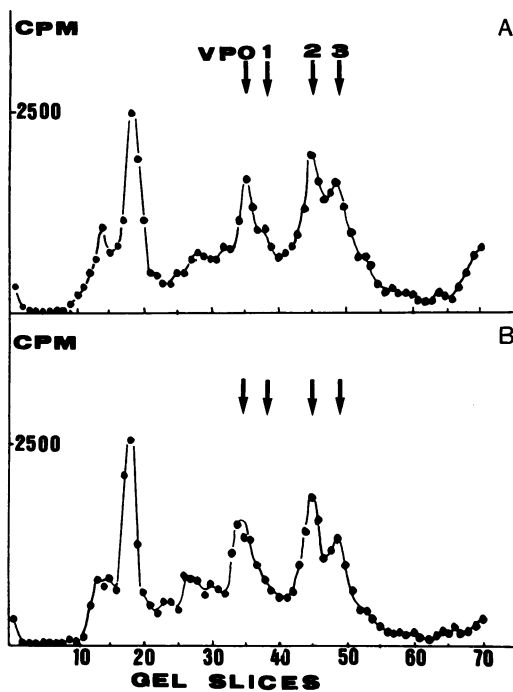
process, then the data reported here suggest that the capsid proteins of the Sabin strain of poliovirus would not have, at high temperature, the configuration required for their ordered aggregation into precursors to virions. The structure once assembled, would be resistant, however, to an elevation in temperature, perhaps as a consequence of protein-protein interactions. The thermosensitivity of the Sabin strain is probably not related to the absence of VP_1 from the procapsids and virions (references 6, 7; Fig. 5) since VP_1 is also absent from a temperature-resistant variant of the Sabin strain (*unpublished results*). Virions from the Sabin strain were not found to be any more thermolabile than those from the Mahoney strain, and the only difference was their greater sensitivity towards SDS treatment (7).

Our results are at variance with those of Garfinkle and Tershak (10) who reported that the defect of the Sabin LSc2ab strain of poliovirus lay in the replication of viral RNA. These authors also analyzed the various polypeptides synthesized in cells infected with the Sabin strain. They showed that, at high temperature, the cleavage of the giant precursor polypeptide, which is the direct product of translation of viral RNA (13), occurred partially, if at all. It is clear from the results presented here that replication of the RNA of our strain is barely thermosensitive, since viral RNA synthesis proceeded, at 38.5 C, at 70% of the 36 C rate (see Fig. 1). In the experiments of Garfinkle and Tershak, the nonpermissive temperature chosen was 39 C. This temperature was deliberately avoided here, because of the phenomenon of thermo-induced degradation of viral RNA (8), which is minimal at 38.5 C, but already quite pronounced at 39 C. As previously discussed (8), this can be a serious cause of misinterpretation of the results of uridine uptake.

Regarding the lack of cleavage of the giant viral precursor polypeptide(s), the discrepancy between the results of Garfinkle and Tershak and ours is not due to the experimental procedures, since we repeated with our strain the same experiment they performed with theirs, using guanidine to synchronize infection and working at 39 C. No alteration of cleavage was detected with our strain under these conditions. Although it cannot be excluded that the discrepancy stems from the difference in the cell lines used, it seems more likely that it is due to a difference in the viral strains studied. The Sabin strain of poliovirus has been cultivated and repeatedly cloned in many laboratories in the last 10 years, and populations of the virus could have evolved differently in different laboratories. The fact that the strain studied by Garfinkle and Tershak is blocked at an earlier step in the processing of viral proteins

than ours suggests that it might bear an additional mutation.

One general conclusion which emerges from this study and the preceding one (8) on the effects of supraoptimal temperatures (15) is that inhibition of viral development at high temperature is the result of two mechanisms. One is cellular and seems to involve the release of lysosomal enzymes from the infected cell. The other is viral, and results from the eventual genetic defect of the viral strain under study. The latter mechanism is often masked by the former and, to be studied, requires that the temperature be kept as low as possible. This duality of the response to elevated temperatures also explains why the same viral strain can exhibit different rt's, depending upon the cell which is used for its growth (14). In turn, this implies that rt's which are higher than the normal temperature for cell growth do not necessarily indicate a true thermosensitivity of the virus, but, more likely, correspond to the triggering of the cellular response. As shown previously (8), replication of all strains of poliovirus, whether thermosensitive or not, is impaired at temperatures above 38.5 C. This is due to release of lysosomal enzymes, which degrade the viral RNAs. Therefore, the fact that, for instance, wild-type poliovirus exhibits an rt value of 40.8 C is probably meaningless in terms of the true level of the virus thermosensitivity.

ADDENDUM IN PROOF

After the present manuscript was submitted for publication, it was reported by Garfinkle and Tershak (Nature N. Biol. 238:206–208) that LSc2ab viral polypeptides were degraded at 39 C. This suggests that lysosomal enzymes were released in their experiment, which is in agreement with our previous observations and the above discussion.

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