Effects of Elevated Temperatures on Mengovirus Ribonucleic Acid Synthesis and Virus Production in Novikoff Rat Hepatoma Cells

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The production of mengovirus in Novikoff rat hepatoma cells is progressively reduced with an increase in incubation temperature of the cells from 34 to 40 C, in spite of the fact that about the same amounts of single-stranded and double-stranded viral ribonucleic acid (RNA) are synthesized at 34, 37, and 40 C; the rate of overall protein synthesis is as high at 40 C as at 37 C. At 40 C, progeny viral RNA accumulates in an undegraded form without being incorporated into virus particles. The results suggest that virus maturation is preferentially inhibited at supraoptimal temperatures. At 42 C, on the other hand, no viral RNA is produced and no viral RNA polymerase activity is detectable in cell lysates. Failure of infected cells to form viral RNA polymerase at 42 C is probably due to an impairment of protein synthesis since most of the polyribosomes are rapidly lost during incubation at 42 C and the rate of amino acid incorporation into protein is 70% lower at 42 C than at 37 C. When infected cells are shifted from 37 to 42 C during the period of active viral RNA synthesis, viral RNA polymerase activity is rapidly lost from the cells, and viral RNA synthesis ceases within 45 min. In contrast, the RNA polymerase is as active *in vitro* at 42 C as at 37 C, and the activity is relatively stable at 42 C.

The question has frequently been raised whether fever is beneficial to the mammalian host in combating viral disease, but a clear answer is not available (3, 5, 9, 10). Nevertheless, studies with mammalian cells in culture have shown that the replication of a number of viruses is inhibited at elevated temperatures at which cellular metabolism is relatively unaffected (2, 7-10, 21-23, 25, 28). For instance, Lwoff (9) reported that the replication of poliovirus in KB cells is maximal at about 35 C and is almost completely inhibited at 39.5 C due to an inhibition of a thermosensitive reaction late in the replicative cycle of the virus. Subsequently, Lwoff (10) reported that the inhibition of viral replication at 39.5 C is due to a reduction in viral ribonucleic acid (RNA) synthesis and the degradation of any newly synthesized viral RNA by a virus-activated ribonuclease. In contrast, Tershak (25) found poliovirus RNA to be stable in HeLa cells at 40 C and concluded that the inhibition of poliovirus replication at 40 C is due to an almost complete inhibition of viral RNA synthesis between 2 and 4 hr after infection.

We observed that mengovirus replication in Novikoff rat hepatoma cells growing in suspension culture was also markedly reduced at 40 C, whereas cellular macromolecular synthesis was about the same at 40 and 37 C. Further studies revealed that the formation of viral RNA polymerase and the synthesis of viral-specific RNA occurred normally at 40 C but that the viral RNA accumulated in the infected cells in an undegraded form without being incorporated into virus particles. At 42 C, on the other hand, no viral RNA polymerase was elicited, and no viral RNA was produced. Further, the viral RNA polymerase activity was highly unstable at 42 C *in vivo*, but not *in vitro*. The results have been presented in a preliminary report (Shea and Plagemann, *Bacteriol. Proc.*, 1970, p. 182).

MATERIALS AND METHODS

The materials and methods used were the same as described in the accompanying report (17). Novikoff rat hepatoma cells (subline N1S1-67) were infected with 10 to 30 plaque-forming units (PFU) per cell.

Infected cells were suspended in basal medium 42 (BM42), except where indicated otherwise. The suspensions were first incubated on a gyratory shaker at 37 C and then transferred to reciprocal water-bath shakers at various temperatures as indicated in the appropriate experiments. Temperature equilibration of 50-ml suspensions occurred within 1 to 2 min. Mouse L cells (subline L-67G) were propagated in suspension culture in Swim's medium 67 as described for N1S1-67 cells (18, 26). The L-67G subline was derived from a line of L cells obtained from Angus F. Graham and had a population-doubling time of about 18 hr.

Plaque assay for mengovirus. Mengovirus concentrations were assayed by plaque assay on L-67G cells by a procedure modified from that of Baker and Glasgow (1). L-67G cells were collected by centrifugation from suspension cultures in the late exponential phase of growth between 10^6 and 1.5×10^6 cells per ml and suspended to 10^8 cells per ml of medium composed of Eagle's basal medium with Earle's salts (Grand Island Biological Co., Grand Island, N.Y.) and 5% (v/v) calf serum. Amounts of 5 ml of this suspension were seeded into 30-ml tissue culture flasks (Falcon Plastics). After complete sheets of cells had formed after 8 to 24 hr of stationary incubation at 37 C, the medium was removed and the flasks were inoculated with 0.2 ml of 10-fold dilutions of mengovirus suspension in BM42 (3 flasks per dilution). After 30 min of incubation at 37 C, 5 ml of Eagle's medium was added per flask and the flasks were incubated stationary for 2 days at 37 C. The medium was poured off, and the cell layer was incubated with 0.5 ml of 20% (v/v) Formalin at room temperature for 30 min and then stained with 0.5 ml of Loeffler's methylene blue for 5 min. The number of plaques obtained by this method was approximately the same as that obtained by a conventional plaque assay with an agar overlay (4).

Assay for viral RNA polymerase. Infected cells were assayed for viral RNA polymerase activity as described previously (13). Briefly, 8×10^6 infected cells were lysed by treatment with sodium deoxycholate in the presence of actinomycin D in a total volume of 0.5 ml. Then the lysate was mixed with 0.4 ml of a solution composed of: 12.5 mM magnesium acetate, 125 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.4), 0.15 mM each of adenosine, cytidine, and uridine triphosphate, 0.025 mM ^3H -guanosine triphosphate (1000 $\mu\text{Ci}/\mu\text{mole}$), 10 mM phosphoenol pyruvate, and 20 μg of pyruvate kinase per ml. The mixtures were incubated at 27 C for 120 min and then analyzed for radioactivity in acid-insoluble material.

Incorporation of labeled amino acids. Suspensions of cells in BM42 were supplemented with ^3H -amino acid mixture (reconstituted protein hydrolysate, Schwarz BioResearch) as indicated in the appropriate experiment. Duplicate 0.5-ml samples of suspension were analyzed for radioactivity in hot acid-insoluble material as described previously (26).

Polyribosome analysis. Samples of about 10^8 cells were harvested by centrifugation and suspended in 3.5 ml of B4 (26) at 0 C. After 10 min, the cells were disrupted by 10 strokes with a Dounce homogenizer (18).

The nuclei were removed by centrifugation at $600 \times g$ for 5 min, and the supernatant fraction was mixed with 0.3 ml of 10% (w/v) sodium deoxycholate and layered onto 26 ml of a linear 0.5 to 1.2 M gradient of sucrose in B2 (13) which was placed over a 2.5-ml cushion of 1.85 M sucrose in B2. The gradients were centrifuged in an SW25 rotor (model L-2 Beckman ultracentrifuge) at 20,000 rev/min, 4 C for 3 hr. The gradients were monitored for absorbancy at 256 nm with a spectrophotometer attached to an ISCO gradient fractionator (13).

RESULTS

Effect of elevated temperatures on viral and cellular RNA synthesis. It has been demonstrated previously (13, 14) that in the presence of 1.25 μg of actinomycin D per ml of suspension, mengovirus-infected N1S1-67 cells incorporate ^3H -uridine mainly into viral-specific RNA. The results in Fig. 1C demonstrate that the virus-induced incorporation of uridine into acid-insoluble material was slightly more rapid at 40 C than at 37 C but that the total amount incorporated was about 40% lower at 40 C than at 37 C. On the other hand, infected cells continuously incubated at 42 C beginning at 0.5 hr after infection failed to incorporate uridine into acid-insoluble material. As indicated by the data in Fig. 1D, uridine incorporation into acid-insoluble material by uninfected cells not treated with actinomycin D was also reduced at elevated temperatures, but there was still significant incorporation at 42 C. The latter results are similar to those obtained with suspension cultures of uninfected HeLa cells (27). The inhibition of uridine incorporation into acid-insoluble material at 42 C by either infected or uninfected cells was probably not due to a failure of the cells to take up or phosphorylate uridine. The data in Fig. 1A and B indicate that the cells incubated at 37, 40, or 42 C incorporated uridine into total cell material (acid-soluble plus acid-insoluble) at about the same rate for 30 to 45 min after addition of the uridine at 2.25 hr. The cells incorporated as much uridine at 42 C as at 40 C, and the total amount incorporated was at least 10 times greater than the amount incorporated into acid-insoluble material. Further, chromatographic analyses of acid-extracts from the cells showed that about 60% of the radioactivity was located in uridine triphosphate (UTP) plus uridine diphosphate (UDP) (mostly UTP), 25% in UDP-sugars and the remainder in uridine monophosphate (UMP) whether the cells were infected or uninfected or incubated at 37, 40, or 42 C (Table 1). Significant amounts of uridine were not present in acid-extracts from cells that had been washed twice with balanced salt solution before acid extraction.

FIG. 1. Incorporation of ^3H -uridine into total cell material and acid-insoluble material by mengovirus-infected (A and C) and uninfected N1S1-67 cells (B and D) at 37, 40, and 42 C. N1S1-67 cells were infected with 20 PFU of mengovirus per cell in BM42 (2×10^6 cells/ml) at 37 C or suspended in BM42 without virus (0 time). After 0.5 hr of incubation at 37 C, portions of each suspension were further incubated at the indicated temperatures in reciprocal water-bath shakers. The suspensions of infected cells were supplemented with actinomycin D to 1.25 μg per ml at 1.5 hr after infection, and all suspensions with 0.01 mM uridine- ^3H (100 $\mu\text{Ci}/\mu\text{mole}$) at 2.25 hr. At various times thereafter, duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in total cell material (A and B) or acid-insoluble material (C and D). All points represent averages of the duplicate samples.

The total amount of uridine incorporated into cells at 40 and 42 C was lower than that incorporated at 37 C (Fig. 1A and B), but the difference varied from 10 to 50% in different experiments. The reduction in the incorporation of uridine into acid-insoluble material at 40 C as compared to 37 C also varied somewhat from experiment to experiment and could have been partially due to the reduction of uridine incorporation into the nucleotide pool.

The loss of total radioactivity from infected cells beginning at about 4 hr after infection coincided temporally with an increase in the number of trypan blue-stainable cells in the suspension (Fig. 2B) and therefore was probably mainly due to virus-induced cell degeneration. This correlation between nucleotide loss from infected cells and cell lysis has been indicated by results from

many experiments and is more clearly illustrated by the results from a pulse-chase experiment presented subsequently (Fig. 7). However, additional factors may have played a role in nucleotide loss from infected cells at 42 C since the loss was generally larger than expected from the proportion of stainable cells in the culture. The nucleotide loss from infected cells seems to be unrelated to the actinomycin treatment of the cells. This is indicated by results from pulse-chase experiments with uninfected cells in which actinomycin D was present during the chase at 37, 40, and 42 C. Actinomycin D did not accelerate the slow loss of label from the nucleotide pool which normally occurs during the chase period either with uninfected or infected cells (Fig. 7; 17).

Effect of elevated temperatures on the formation of viral RNA polymerase and virus-induced cell death. The results of the following experiment indicate that the failure of infected cells to produce viral RNA at 42 C was probably due to a lack of viral-induced RNA polymerase. Samples of a suspension of infected cells were incubated at 37, 40, and 42 C, and samples thereof were assayed *in vitro* for viral RNA polymerase activity (Fig. 2A). Other samples were monitored for cell degeneration by vital staining with trypan blue (Fig. 2B). No viral-induced RNA polymerase activity was detectable in cells incubated at 42 C (Fig. 2A) and virus-induced cell degeneration was almost completely prevented (Fig. 2B). On the other hand, about the same amount of RNA polymerase activity was formed between 1.5 and 4 hr after infection at 40 C as at 37 C (Fig. 2A). Polymerase activity began to increase 0.5 hr earlier at 40 C than at 37 C and at a greater rate. Infected cells also began to degenerate earlier at 40 C than at 37 C (Fig. 2B). The secondary increase in RNA polymerase at 37 C, which occurred during the period of cell degeneration between 4 and 6 hr, does not represent *de novo* synthesis of polymerase, since it occurs in the absence of protein synthesis (14). It seems to represent an activation of polymerase or RNA template incidental to cell degeneration (13). Only a small secondary increase was observed in cells incubated at 40 C.

Inhibition of protein synthesis at elevated temperatures. The results of the following experiments suggest that the lack of formation of viral RNA polymerase at 42 C might have been due to an impairment of protein synthesis at this temperature. Infected cells were incubated in BM42 at 37 C until 1 hr after infection. Then portions of the suspension were further incubated at 37, 40, and 42 C, and samples thereof were monitored for incorporation of labeled amino acids into hot acid-insoluble material (Fig. 3A). Other samples

TABLE 1. Distribution of ^3H among uridine nucleotides in acid-extracts from infected and uninfected cells labeled with uridine at various temperatures^a

Cells	Temp (C)	UTP		UDP-sugars		UMP	
		Counts/min ^b	Per cent ^c	Counts/min ^b	Per cent ^c	Counts/min ^b	Per cent ^c
Uninfected	37	31,630	60	10,110	20	10,840	20
	40	30,970	58	12,170	24	9,480	18
	42	39,000	58	17,700	26	10,090	16
Infected	37	30,270	55	12,450	23	12,130	22
	40	33,900	56	15,810	26	10,940	18
	42	32,210	59	14,480	26	8,220	15

^a Details of the experiment are described in the legend to Fig. 1. After 150 min of labeling with ^3H -uridine at the indicated temperatures, samples of 1.5×10^7 uninfected or infected cells were collected by centrifugation, washed once in basal salt solution, and extracted with perchloric acid; the acid extracts were analyzed by ascending paper chromatography as described previously (20).

^b Total amount of radioactivity associated with the indicated components after chromatography of 50 μl of acid-extract. UDP was not well separated from UTP and since UDP contained little radioactivity it was included in the UTP fraction.

^c Per cent of total radioactivity in nucleotides.

FIG. 2. Viral RNA polymerase activity (A) and virus-induced cell degeneration (B) as a function of time of incubation at 37, 40, and 42 C. Samples of a suspension of infected cells in BM42 (2×10^6 cells/ml) were incubated at the indicated temperatures in reciprocal water-bath shakers beginning at 0.5 hr after infection. Samples of cells were assayed for viral RNA polymerase activity and cell degeneration by vital staining with trypan blue as described in the text. Points for polymerase activity represent averages of duplicate determinations.

of cells were fractionated and the cytoplasmic fractions were analyzed for polyribosome content by centrifugation through sucrose density gradients (Fig. 3B). The data in Fig. 3 show that the rate of amino acid incorporation at 42 C was only

about 30% of that at 37 C and that incubating the cells at 42 C resulted in a rapid loss of the polyribosomes. At 1 hr after infection over 90% of the ribosomes of the cell were located in polyribosomes, but within 10 min of incubation of the cells at 42 C most of the polyribosomes had disaggregated with the concomitant accumulation of 80 and 110S ribosomal structures (Fig. 3B). In contrast, at 40 C the rate of amino acid incorporation was about 30% higher than at 37 C (Fig. 3A), and there was no significant change in the polyribosome content of the cells (*not shown*). Results from additional experiments have shown that the loss of polyribosomes at 42 C was largely prevented by addition of cycloheximide (50 $\mu\text{g}/\text{ml}$) to the cell suspension just prior to shifting it to 42 C. These results are consistent with the hypothesis (12) that the rapid loss of polyribosomes at 42 C is due to an inhibition of the reattachment of ribosomes to messenger RNA, that is of polypeptide chain initiation, while polypeptide chain elongation and completion continue. This hypothesis is supported by the finding that the residual polyribosomes of cells incubated at 42 C were very small although they were at least as active in polypeptide synthesis as the larger polyribosomes present at 37 C. The latter conclusion is indicated by the fact that the number of ribosomes associated with polyribosomes was reduced 80 to 85% by shifting the cells from 37 to 42 C, whereas the rate of amino acid incorporation was only reduced about 70% (compare Fig. 3A and B).

Uninfected N1S1067 cells (Shea and Plagemann, *in preparation*) and HeLa cells (12) incubated at 42 C in growth medium have the capacity to recover partially from the impairment of poly-

FIG. 3. Incorporation of amino acids by infected N1S1-67 cells at 37, 40, and 42°C (A) and disaggregation of polyribosomes at 42°C (B). A suspension of infected cells in BM42 (2×10^6 cells/ml) was incubated at 37°C until 1 hr after infection. (A) Then samples of the suspension were shifted to the indicated temperatures and supplemented with 1 μCi of ^3H -reconstituted protein hydrolysate per ml. At various times thereafter, duplicate 0.5-ml samples were analyzed for radioactivity in hot acid-insoluble material. Points represent averages of the duplicate samples. (B) After 1 hr of incubation at 37°C, a cytoplasmic fraction was prepared from 10^8 cells, treated with deoxycholate and analyzed by sucrose gradient centrifugation as described in the text. The remainder of the suspension was further incubated at 42°C, and cytoplasmic extracts were prepared at 10, 30, and 60 min and analyzed by sucrose gradient centrifugation. Conditions of centrifugation: 0.5 to 1.2 M sucrose gradient, 20,000 rev/min, SW25 rotor, 4°C, 3 hr.

peptide chain initiation during continued incubation at 42°C, since the rapid loss of polyribosomes at 42°C is followed by a partial reformation of the polyribosomes. Such recovery at 42°C was not observed with infected N1S1-67 cells incubated in BM42. The rate of amino acid incorporation remained low at 42°C (Fig. 3A), and the sedimentation profiles of cytoplasmic extracts from cells at 30 and 60 min of incubation at 42°C were very similar to that of the 10-min extract (Fig. 3B).

Shift-down and shift-up experiments. In the following experiment, a suspension of infected cells was incubated at 42°C and samples thereof were shifted to 37°C at 2 and 3 hr after infection. The results in Fig. 4 demonstrate that the formation of viral RNA polymerase and the beginning of viral RNA synthesis were delayed for about 1 hr after the temperature shift, indicating that the early events that lead to the appearance of polymerase activity did not occur at 42°C. A similar delay in RNA polymerase formation is observed when protein synthesis is inhibited during the first

2 to 3 hr after infection (14). Further, the results indicate that the capacity of the infected cells to produce RNA polymerase and viral RNA became progressively reduced with time of incubation at 42°C. A similar observation has been made with poliovirus-infected KB cells (10) and might result from a general adverse effect on the cells, since macromolecular synthesis of uninfected N1S1-67 cells was also irreversibly damaged by continued incubation at 42°C. When uninfected cells were incubated in BM42 at 42°C and then shifted back to 37°C after 2 hr at 42°C, the rates of uridine and amino acid incorporation into acid-insoluble material were both reduced 60 to 70%, when compared to cells incubated continuously at 37°C (not shown).

When cells were infected at 37°C and then shifted to 42°C during the period of active viral RNA synthesis at 3 hr after infection, the rate of uridine incorporation into viral-specific RNA decreased immediately and RNA synthesis ceased completely within 45 min after the temperature shift (Fig. 5A). Little radioactivity was subsequently lost from acid-insoluble material. To

FIG. 4. Effect of shifting infected cells from 42 to 37°C at various times after infection on viral RNA synthesis (A) and formation of viral RNA polymerase (B). One portion of a suspension of infected cells in BM42 (2×10^6 cells/ml) was continuously incubated at 37°C, and two other portions were incubated at 42°C beginning at 0.5 hr after infection. One of the latter two suspensions was shifted to 37°C at 2 hr and the other at 3 hr after infection. (A) Samples of each suspension were supplemented with actinomycin D (1.25 $\mu\text{g}/\text{ml}$) at 1.5 hr and with 0.01 mM of uridine- ^3H (100 $\mu\text{Ci}/\mu\text{mole}$) at 2 hr and subsequently monitored for radioactivity in acid-insoluble material. (B) Other samples of each suspension were assayed for viral RNA polymerase activity as described in the text. All points represent averages of duplicate determinations.

FIG. 5. Effect of shifting uninfected and infected cells from 37 to 42 C at 3 hr on cellular and viral RNA synthesis, respectively (A), and viral RNA polymerase activity (B). (A) Suspensions of uninfected and infected cells in BM42 (2×10^6 cells/ml) were supplemented with 0.01 mM uridine-5- ^3H (100 $\mu\text{Ci}/\mu\text{mole}$) at 2 hr. The suspension of infected cells was also supplemented with 1.25 μg of actinomycin D per ml at 1.5 hr. Duplicate 0.5-ml samples of each suspension were analyzed for radioactivity in acid-insoluble material. (B) A suspension of infected cells in BM42 was incubated on a gyratory shaker at 37 C until 3 hr after infection. Then one half of the suspension was further incubated at 37 C and the other half at 42 C in reciprocal water-bath shakers. Samples of 8×10^6 cells of the suspensions were assayed for viral RNA polymerase as described in the text. All points represent averages of duplicate determinations.

ascertain whether some of the viral RNA synthesized at 37 C became degraded to low-molecular-weight, acid-insoluble fragments during incubation of the cells at 42 C, samples of cells were removed at various times, and treated with sodium dodecyl sulfate; the RNA was analyzed by centrifugation through sucrose density gradients. The radioactivity profiles were about the same in all samples, most of the radioactivity being located in 35S viral RNA and very little in low-molecular-weight RNA (13). The results indicate that little degradation of viral RNA, if any, occurred at 42 C. The results from another type of experiment support this conclusion. Infected cells were treated with actinomycin D at 1.5 hr after infection and pulse-labeled at 37 C with ^3H -uridine from 2 to 2.5 hr. The cells were then collected by centrifugation, washed free of residual labeled uridine, and then further incubated at 42 C in BM42 containing an excess of unlabeled uridine (1 mM). At various times during the chase at 42 C, RNA was extracted from samples of cells and analyzed by sucrose density gradient centrifugation. Again no significant degradation of viral RNA was detected during a 2-hr chase at 42 C.

Shifting uninfected cells (not treated with ac-

tinomycin D) from 37 C to 42 C also resulted in a decrease in the rate of uridine incorporation into acid-insoluble material after 15 to 20 min of incubation at 42 C, but incorporation at 42 C continued longer than in infected cells (Fig. 5A).

The results in Fig. 5B demonstrate that the depression of viral RNA synthesis after the shift to 42 C at 3 hr after infection correlated with a rapid loss of viral RNA polymerase activity as measured in cell-free preparations. About 90% of the activity was lost after 1 hr of incubation of the cells at 42 C. A similar loss of viral RNA polymerase activity was observed whether the activity was assayed in cell lysates (Fig. 5B) or in mitochondrial-microsomal fractions prepared from infected cells after shifting the culture to 42 C or whether samples of the mitochondrial-microsomal fractions were assayed for polymerase activity at 27 or 37 C (not shown). In contrast, the results in Fig. 6 demonstrate that the RNA polymerase activity of a cytoplasmic fraction from 4-hr infected cells was as high at 42 C as at 37 or 27 C. Further, preincubation of the cytoplasmic fraction at 42 C for 30 min reduced its subsequent activity at 37 and 42 C only about 15 and 25%, respectively (not shown).

FIG. 6. Time course of *in vitro* viral RNA polymerase reaction at 27, 37 and 42°C. A mitochondrial-microsomal fraction was prepared from 4-hr infected cells as described previously (19). One volume of this fraction containing 17 mg of protein per ml was mixed with two volumes of polymerase reaction solution described in the text, and portions thereof were incubated at 27, 37, or 42°C. Duplicate 0.8-ml samples were removed as a function of time of incubation, immediately mixed with 0.5 ml of 0.1 M pyrophosphate and frozen in a bath of CO₂ in ethanol, and later analyzed for radioactivity in acid-insoluble material. All points represent averages of the duplicate samples.

Effect of temperature on virus production. In repeated experiments it was observed that although viral RNA synthesis appeared to be approximately normal at 40°C (Fig. 1), the yield of virus was reduced 70 to 80% at this temperature. Representative results are presented in Table 2. The effect was confirmed by a biochemical analysis of virus production at various temperatures. In one type of experiment, samples of actinomycin-treated infected cells were continuously incubated with ³H-uridine at 37 and 40°C and samples of cells were analyzed for radioactivity in single-stranded and double-stranded viral RNA and in mature virus. The results showed that the amount of uridine incorporated into viral-specific RNA was only slightly less at 40°C than at 37°C (see Fig. 1), whereas the amount of radioactivity incorporated into virus was 60% lower at 40°C than at 37°C (not shown).

In another type of experiment, actinomycin-treated infected cells were harvested after 2.5 hr of

incubation at 37°C and then labeled with ³H-uridine at 6°C for 30 min. This procedure leads to a labeling of the uridine nucleotide pool without simultaneous incorporation of label into viral RNA (17; see Fig. 7). Then the cells were collected by centrifugation and washed free of residual uridine; samples of the cells were further incubated in BM42 at 34, 37, 40, and 42°C. The results in Fig. 7A demonstrate that the rate at which label was chased from the nucleotide pool into acid-insoluble material increased as the temperature was increased from 34 to 37 to 40°C. On the other hand, label ceased to be chased the earlier the higher the temperature. This finding might have been related to the observation (Fig. 7B) that the infected cells degenerated more rapidly as the temperature was increased from 34 to 40°C. The loss of radioactivity from the nucleotide pool at 37 and 40°C was probably also due to virus-induced cell lysis, since it correlated with an increase in the number of trypan blue-stainable cells in the cultures (compare Fig. 7A and B). At 42°C, incorporation into acid-insoluble material ceased during the first 45 min of the chase, in spite of the fact that the total amount of radioactivity associated with the cells was little affected (Fig. 7A) and only few cells degenerated (Fig. 7B).

During the chase period, samples of the suspensions were also analyzed for radioactivity in single-stranded and double-stranded viral RNA and in virus (Fig. 8). The time courses of chase of label into single-stranded RNA at the different temperatures was about the same as already described for total acid-insoluble material, since most (70 to 80%) of the label in acid-insoluble material represents free single-stranded viral RNA (14; see Fig. 8). A comparison of the data in

TABLE 2. Effect of elevated temperatures on virus production^a

Time after infection (hr)	PFU/ml ^b at			HA units/ml at		
	37°C	40°C	42°C	37°C	40°C	42°C
2	2.3	2.0	2.0	160	160	160
3	19	3.0		640	320	
4	30	20		10,280	5,240	160
6	160	38	2.8	20,560	5,240	320

^a Details of the experiment are described in the legend to Fig. 1. At the indicated times, samples of suspension were frozen in a bath of CO₂ in ethanol. Later the samples were thawed, mixed with sodium deoxycholate to 0.5% (w/v), and analyzed for virus concentration by plaque assay and HA titration as described in the text.

^b Values to be multiplied by 10⁶.

FIG. 7. Labeling of infected cells with ^3H -uridine at 6 C and subsequent chase into viral-specific RNA (A) and cell degeneration (B) at various temperatures. A suspension of infected cells in medium 67 (2×10^6 cells/ml) was incubated at 37 C and supplemented with actinomycin D (1.25 $\mu\text{g}/\text{ml}$) at 2 hr after infection. At 2.5 hr, the cells were collected by centrifugation and suspended in cold (6 C) BM42 containing 0.5 μM uridine- ^3H (4,000 $\mu\text{Ci}/\mu\text{mole}$) and further incubated at 6 C for 30 min. Then the cells were collected by centrifugation at 0 C and washed once in cold BM42. Samples of the cells were suspended in BM42 that had been equilibrated at the indicated temperatures. (A) Duplicate 0.5-ml samples of suspension were analyzed (i) for total radioactivity associated with the cells (acid-soluble plus acid-insoluble), and (ii) for radioactivity in acid-insoluble material. Points represent averages of the duplicate samples. Other samples of suspension were analyzed as described in the legend to Fig. 8 and (B) for cell degeneration by vital staining with trypan blue.

Fig. 8A and B shows that the synthesis of double-stranded RNA varied with temperature in the same manner as the synthesis of single-stranded RNA. In contrast, as indicated by the data in Fig. 8C, incorporation of label into virus was highest at 34 C, in spite of the fact that more viral RNA was produced at 37 and 40 C than at 34 C (Fig. 8A and B). Only about 40% as much label was chased into virus at 40 C as at 37 C, and none was incorporated into virus at 42 C. At 6.5 hr after infection the virus titers were 5,120, 2,560, 1,230 and 10 hemagglutination (HA) units and 7.5×10^8 , 2.8×10^8 , 6×10^7 and 2×10^6 PFU per ml at 34, 37, 40, and 42 C, respectively. The beginning of virus production (Fig. 8C) and of cell degeneration (Fig. 7B) was delayed about 1 hr in this experiment because of the 30-min incubation of the cells at 6 C and their repeated sedimentation.

DISCUSSION

Our finding that the production of mengovirus in N1S1-67 cells is markedly reduced at 40 C (Table 2; Fig. 8) is similar to that reported for poliovirus replication in KB (9, 10) or HeLa cells (25). However, in contrast to the results with poliovirus (10, 25), reduced mengovirus replication at 40 C is not due to a decreased synthesis of viral-specific RNA or to a degradation of the viral RNA but rather to a failure of the progeny viral RNA to be incorporated into mature virus particles (Fig. 8). The amount of progeny virus produced is even higher at 34 C than at 37 C, in spite of the fact that more viral-specific RNA is synthesized at 37 C than at 34 C (Fig. 8). The optimum temperature of wild-type poliovirus production also appears to be around 34 C (10, 25). The reason for the decreased incorporation of mengovirus RNA into virus particles above 34 C is

FIG. 8. Chase of uridine into single-stranded (A) and double-stranded viral RNA (B) and into virus (C) at various temperatures in infected cells prelabeled at 6 C. The details of the experiments are described in the legend to Fig. 7. Actinomycin-treated, infected cells were labeled with ³H-uridine at 6 C from 2.5 to 3 hr after infection, freed of excess labeled uridine, and further incubated at the indicated temperatures. During the chase, duplicate 0.5-ml samples of suspension were analyzed for total radioactivity in acid-insoluble material (ii; see Fig. 7). Other duplicate samples were analyzed as described previously (14, 17) for: (iii) radioactivity in acid-insoluble material after treatment of samples with ribonuclease in the presence of sodium deoxycholate; and (iv) for radioactivity in acid-insoluble material after heating the samples at 70 C for 1 hr and treatment with ribonuclease plus deoxycholate. Radioactivity in single-stranded RNA and double-stranded viral RNA and in virus was calculated (14, 17) as follows: single-stranded RNA = radioactivity in (ii) minus that in (iii); RNA in virus = (iii) minus (iv); and double-stranded = (iv).

unknown. Although the sedimentation profiles of viral-specific RNA synthesized at 37, 40 or 42 C are all about the same, it cannot be ruled out that minor structural differences exist between RNA molecules synthesized at different temperatures which might affect the efficiency of their incorporation into virus particles. Other possibilities are that a capsid protein might be unstable at temperatures above 34 C or that the maturation process itself is affected. The synthesis of structural protein *per se* is probably not adversely affected at temperatures up to at least 40 C, since the rates of overall protein synthesis and polyribosome integrity are about the same at 37 and 40 C. The inhibition of production of infectious adenovirus (21, 28) or vaccinia virus (8) at supra-optimal temperatures seems also to be due to the inhibition of virus maturation or the lack of a structural protein. However, the decrease in overall virus yield with increase in temperature from

34 to 40 C could also be related to the fact that the infected cells begin to degenerate earlier as the temperature is increased (Fig. 2, 7).

The results also demonstrate that the differences in the amounts of uridine incorporated into viral-specific RNA and into virus at various temperatures were not due to differences in the rates of uridine uptake or phosphorylation since the time courses of incorporation of uridine into the nucleotide pool were very similar at 37, 40, and 42 C (Fig. 1). These differences were also observed when the nucleotide pool was prelabeled at 6 C and the incorporation of label into RNA and virus was measured during a subsequent chase at the various temperatures (Fig. 8). Incorporation of uridine into cellular RNA is also more affected by changes in temperature than is the incorporation of uridine into the nucleotide pool (Fig. 1; 16).

On the other hand, virus replication seems to

be inhibited at 42 C because no active viral RNA polymerase is formed in infected cells (Fig. 2) and consequently no viral RNA (Fig. 1). There are many possible causes for the failure of infected cells to produce functional RNA polymerase at 42 C. The most likely explanation is that it is related to the inhibition of protein synthesis. Even though N1S1-67 cells continue to incorporate amino acids into protein at a reduced rate at 42 C, there is no certainty that the protein that is synthesized is not defective. For instance, the impairment of polypeptide chain initiation might result in the production of faulty proteins. Evidence has been presented that the marked loss of polyribosomes at 42 C is due to an inhibition of the attachment of ribosomes to messenger RNA (12). Phenethyl alcohol (15), butanol (6), ethionine (24), and NaF (11) have a similar effect on protein synthesis by mammalian cells.

It is also possible, however, that no RNA polymerase is formed at 42 C because the translation of viral RNA is preferentially inhibited at 42 C. This possibility is difficult to analyze since mengovirus infection of N1S1-67 cells has little effect on cellular protein synthesis (13) and the amino acids incorporated by infected cells are therefore probably incorporated primarily into cell protein. Other possibilities are that RNA polymerase is produced at 42 C but is inactivated as rapidly as it is synthesized, or that it does not become functional due to a failure to become associated with its RNA template. The latter possibilities are suggested by the finding that shifting the cells to 42 C at later stages of the virus replication cycle results in a rapid loss of viral RNA polymerase activity and consequently in the cessation of viral RNA synthesis (Fig. 5). This loss in polymerase activity is probably not due to a thermal inactivation of the RNA template-RNA polymerase complex since the polymerase is as active *in vitro* at 42 C as at 37 C and is relatively stable at 42 C (Fig. 6). It is also probably not due to a degradation of the RNA template since viral RNA seems to be stable in infected cells incubated at 42 C. A similar loss of polymerase activity is observed after treatment of infected cells with puromycin at 3 to 4 hr after infection (19). The reason for this apparent metabolic instability of the viral RNA polymerase *in vivo* is not known. It seems to be due to a metabolic process operating in whole cells but not in cell-free cytoplasmic fractions. Loss of polymerase activity could be due to a dissociation of polymerase and RNA template, to an inactivation of polymerase *per se*, or to a loss of a protein factor required for its activity. The replication of variola virus (2) and influenza virus (22) seems to be inhibited at elevated temperatures due to an inactivation of the

DNA and RNA polymerases, respectively, but the polymerases induced by these viruses are heat labile both *in vivo* and *in vitro*.

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