

# RNA METABOLISM IN HeLa CELLS AT REDUCED TEMPERATURE

## I. Modified Processing of 45S RNA

RONALD H. STEVENS and HAROLD AMOS

From the Department of Microbiology and Molecular Genetics, Harvard Medical School,  
Boston, Massachusetts 02115

### ABSTRACT

Incubation of HeLa cells at suboptimal temperature has been used to study the synthesis of 45S ribosomal RNA precursor and the individual steps of the subsequent processing to 28S RNA. Below 20°C no detectable 45S RNA is formed. The processing of 45S RNA to 32S RNA ceases around 15°C, and the processing of 32S RNA to 28S RNA is inhibited near 25°C. Prolonged incubation at reduced temperature results in further modification of the processing, resulting in the apparent accumulation of 41S RNA. The products of these reactions at reduced temperature appear normal in that the ribosomal RNA made at 27°C can be isolated from functional polyribosomes in the cytoplasm after a short incubation at 37°C.

### INTRODUCTION

Evidence from a considerable number of experiments has confirmed the existence, in mammalian cells, of a high molecular weight ribosomal RNA precursor (45S) and the subsequent processing of it to stable ribosomal RNA (1-4). 45S RNA is synthesized in the nucleolus (5), where it is methylated (6-7), and associated with preribosomal proteins (8). It has a lifetime of several minutes before being cleaved to produce 41S RNA (9), which in turn is rapidly converted to a 32S RNA and an 18S RNA molecule (10). While still in the nucleolus, the 32S RNA gives rise to a 28S RNA molecule which passes through the nucleoplasm and enters the cytoplasm as a nearly mature ribosomal subunit approximately 1 hr after the completion of the 45S precursor (11). During the processing, over 40% of the RNA originally pres-

ent in the 45S RNA is lost by degradation. This degraded RNA is characterized by a high guanine-cytosine (GC) content (12-13) and contains essentially no methylated bases (10).

While the sequence of reactions in the processing is well known, very little information has been obtained concerning the individual steps. Incubation of cells at suboptimal temperatures has been employed in an effort to accumulate intermediates, with the objective of focusing on a single step in the processing. We have found that the different steps of the processing can be differentially reduced by incubation at suboptimal temperatures. This results in the accumulation of 32S RNA and, under conditions of prolonged exposure to low temperatures, of 41S and possibly 36S as well. The formation of 18S RNA will be considered at a later time.

## MATERIAL AND METHODS

### Cells

The HeLa cells used were a mycoplasma-free strain obtained from Microbiological Associates Inc., Bethesda, Md., and were checked periodically for mycoplasma contamination by radioautography with thymidine-<sup>3</sup>H (14). The cells were routinely grown as confluent monolayers in Eagle's Minimal Essential Medium (MEM) (15) supplemented with 10% calf serum. Before each experiment, the medium was removed and the monolayers were covered with 3.0 ml of 0.13% trypsin in balanced saline solution for 10 min. The bottles were shaken to dislodge the cells, and 5.0 ml of fresh medium was added. The suspended cells were removed from the bottles, pooled, and centrifuged for 5 min at 500 *g* at 1°C. The cell pellet was then resuspended in the experimental medium. The effect of the trypsinization appeared to be negligible, as cells grown in suspension culture gave similar results when substituted for monolayer cultures.

### Isolation of RNA

The possibility existed that the RNA produced at reduced temperatures would be more labile than normal RNA. Therefore a method for extraction of RNA which did not require organic solvents was sought. The cells were washed twice with 10 ml of RSB (0.01 M NaCl, 0.01 M Tris-HCl, 0.0015 M MgCl<sub>2</sub>, pH 7.4); the pellet from the second wash was resuspended in 1 ml RSB to which 1 ml of a solution of 7.5% sodium dodecyl sulfate (SDS) and 0.05 M ethylenediaminetetraacetic acid (EDTA) was added. The DNA was sheared by several strokes in a Dounce homogenizer. After chilling the suspension for 30 min in an ice bath, the precipitate was removed by filtration through a Millipore filter (Type HA) (Millipore Corp., Bedford, Mass.). Any material that remained on the surface of the tube was recovered by a 1 ml rinse with RSB. The filtrate was, in turn, chilled for 30 min and filtered again through a Millipore filter. RNA was precipitated from the filtrate by the addition of 2 vol of cold ethanol. The yield of RNA with this method has proved to be highly reproducible. The A<sub>260</sub>/A<sub>280</sub> was consistently above 1.85.

### Electrophoresis

Electrophoresis was performed according to Weinberg et al. (9) with ethylene diacrylate as a cross-linking agent. After electrophoresis of the RNA (10 µg/gel) at 5 ma/gel for the time stated in each experiment, the gels were sliced into 1-mm sections and dissolved by the addition of 0.2 ml of 1 M

piperidine, before the addition of Bray's scintillation fluid (16). Radioactive counts of gel slices were monitored in a Packard liquid scintillation counter, model number 3375.

### Cell Fractionation and Polysome Extraction

Polyribosomes were extracted and analyzed on sucrose gradients as described by Penman et al. (17).

### Isolation of Uridine Nucleotides

The method of Salser et al. (18) was used for the isolation and determination of radioactivity of soluble nucleotides. Labeled cells were precipitated in 5 ml of 0.75 M perchloric acid, and after 2 hr at 4°C the precipitates were removed by centrifugation for 15 min at 3000 *g*. The perchloric acid-soluble material was adsorbed onto 50 mg of activated charcoal, and the charcoal was washed three times with 5 ml of 0.001 M HCl. The nucleotides were eluted from the charcoal with 2 ml of 95% ethanol, 1 M NH<sub>4</sub>OH, H<sub>2</sub>O (66:10:133 by volume), and the radioactivity was determined on a 0.2 ml sample of the mixture in 10 ml of Bray's scintillation fluid (16).

### Radioisotopes

L-methyl-<sup>3</sup>H-methionine (3.3 Ci/mmol), 2-uridine-<sup>14</sup>C (37 mCi/mmol), and serine-<sup>14</sup>C (140 mCi/mmol) were purchased from Schwarz Bio Research, Orangeburg, N. Y. 5-uridine-<sup>3</sup>H (20 Ci/mmol) and H<sub>3</sub><sup>32</sup>PO<sub>4</sub>, carrier free, were purchased from New England Nuclear Corp., Boston, Mass.

## RESULTS

### Synthesis and Methylation of Ribosomal

#### Precursor RNA

For studies involving ribosomal RNA, the use of methyl-labeled methionine has an advantage over radioactive uridine in that the 45S precursor RNA becomes highly labeled while little radioactive heterogeneous nuclear RNA is formed (6-7). Furthermore, the methyl groups are conserved during the processing of 45S RNA, allowing the labeled 28S and 18S RNA molecules to be equated with the labeled 45S precursor RNA (6, 10). Finally, with labeled methionine it is possible to perform pulse-chase experiments which are difficult to accomplish with radioactive uridine due to the extensive turnover of the heterogeneous nuclear RNA (Hn RNA) (19, 20), as well as the slow equilibration of the nucleotide pools (21).

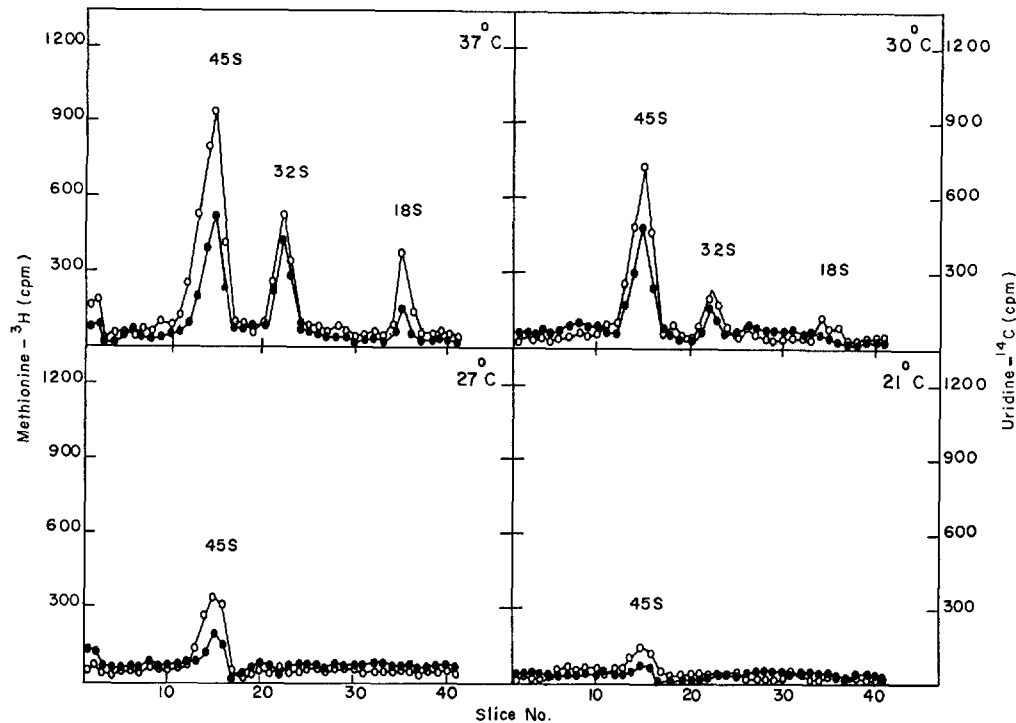


FIGURE 1 Synthesis and methylation of ribosomal RNA precursors.  $5 \times 10^7$  cells were harvested and resuspended in 5 ml of medium containing  $1 \mu\text{Ci/ml}$  uridine- $^{14}\text{C}$  ( $\circ$ ) and  $60 \mu\text{Ci/ml}$  methionine- $^3\text{H}$  ( $\bullet$ ). Incubation was performed at the given temperature for 40 min with gentle periodic agitation. The RNA was extracted and electrophoresis was carried out on 2.6% gels for 6 hr.

TABLE I  
Synthesis and Methylation of 45S RNA

Temperature	$^{14}\text{C}$	$^3\text{H}$	$^3\text{H}/^{14}\text{C}$	$^{14}\text{C}$ % of control	$^3\text{H}$ % of control
	<i>cpm</i>	<i>cpm</i>			
37°C (control)	2305	3675	1.60	100	100
30°C	1258	2110	1.70	53	57
27°C	465	800	1.72	20	21
21°C	155	360	1.54	6	9

The uridine- $^{14}\text{C}$  counts per minute and the methionine- $^3\text{H}$  counts per minute in the 45S, 32S, and 18S RNA peaks in Fig. 1 were summed for each temperature and expressed as the ratio of  $^3\text{H}/^{14}\text{C}$  counts per minute and as per cent of the 37°C counts per minute.

The use of radioactive methionine for the study of ribosomal RNA metabolism at suboptimal temperatures required that, as the culture temperature is reduced, the activity of the methylating enzyme not become limiting before that of the RNA polymerase. In order to determine the rela-

tive activity of the two enzymes as the temperature is lowered, cells were incubated for 40 min at 37°, 30°, 27°, and 21°C in the presence of uridine- $^{14}\text{C}$  and L-methyl- $^3\text{H}$ -methionine. In this, and subsequent experiments utilizing radioactive methionine, medium lacking methionine and con-

taining adenosine ( $10^{-5}$  M) and guanosine ( $10^{-5}$  M) was used to prevent significant incorporation into the RNA of purines synthesized in the presence of the labeled methionine. The RNA was extracted and electrophoresis was performed as described in Materials and Methods. The results are shown in Fig. 1. The species of RNA labeled at different temperatures with uridine- $^{14}$ C showed similar labeling patterns with the L-methyl- $^3$ H-methionine at the several temperatures. Expressed in a different fashion in Table I, the total label present as uridine- $^{14}$ C counts, corrected for losses due to the processing of the RNA, is compared with the  $^3$ H counts which represent methylation. As the temperature was reduced, the ratio of  $^3$ H/ $^{14}$ C counts was not significantly altered, suggesting that it is the RNA polymerase and not the methylase which becomes limiting as the temperature is reduced.

#### Uridylate Pool at Lowered Temperatures

Our interpretation of the effect of temperature on rRNA synthesis assumes that effects on the uptake of radioactive uridine are not the limiting factor. To rule this out, the equilibration of the acid-soluble uridine nucleotide pool was determined at 37°, 30°, 25°, 20°, and 15°C. Cells were labeled for 5, 10, 20, 45, and 75 min at the corresponding temperature with uridine- $^3$ H, and the acid-soluble nucleotides were extracted as de-

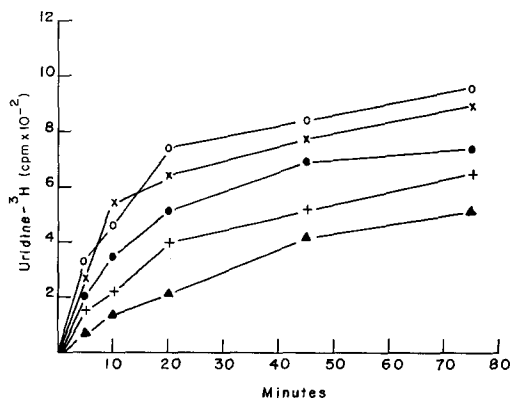


FIGURE 2 Labeling of the uridine nucleotide pool at reduced temperature.  $2 \times 10^6$  cells in 5 ml of MEM were incubated at each temperature in the presence of  $10 \mu\text{Ci/ml}$  uridine- $^3$ H. Samples (0.5 ml) were removed at the indicated times. The acid-soluble nucleotides were determined as described in Materials and Methods. ○, 37°C; ×, 30°C; ●, 25°C; +, 20°C; ▲, 15°C.

scribed in Materials and Methods. With temperature reduction, the equilibration of the acid-soluble pool was not sufficiently affected to account for the reduced rRNA synthesis observed at those temperatures (Fig. 2).

#### Limiting Temperature for 45S Synthesis

To obtain a more accurate determination of the temperature at which the synthesis of 45S RNA begins, portions of a cell suspension were incubated at 37°, 33°, 30°, 26°, 24°, and 19°C for 3 hr in the presence of L-methyl- $^3$ H-methionine. The RNA was then extracted and analyzed on acrylamide gels. Fig. 3 shows that the amount of ribosomal RNA synthesis decreased with reduced temperatures. At 27°C, the peak corresponding to 45S RNA is greatly reduced, yet accumulation of label into 32S and 28S RNA molecules indicates that the 45S formed is processed to 32S RNA. At 24°C, 32S RNA accumulates but no labeled 28S RNA is detected. The absence of the 28S RNA peak and the accumulation of label into 32S RNA indicates that the processing of 32S RNA to 28S RNA is significantly reduced at approximately 24°C. At 19°C there are no detectable peaks, suggesting that 45S RNA is no longer being synthesized. In order to quantitate the results, the counts in each of the species of RNA are summed for each temperature and plotted as the percentage of the control culture at 37°C (Fig. 4). Extrapolating from the linearity observed over the initial portion of the curves, the synthesis of 45S RNA would be inhibited below 20°C, as would the processing of 45S RNA to 32S RNA, while the processing of 32S RNA to 28S RNA would be inhibited nearer 25°C.

#### Limiting Temperature for 32S RNA Formation

While the measurement of the synthesis of 45S RNA can be performed by continuous labeling, the study of the processing reactions by this method assumes that the 45S RNA produced at suboptimal temperatures is normal and in sufficient quantity to allow the subsequent processing.

In order to measure the processing of 45S RNA to 32S RNA, the 45S RNA was made at 37°C and the processing to 32S RNA was observed at the reduced temperatures. A population of cells was incubated in methionine-free medium at 37°C for

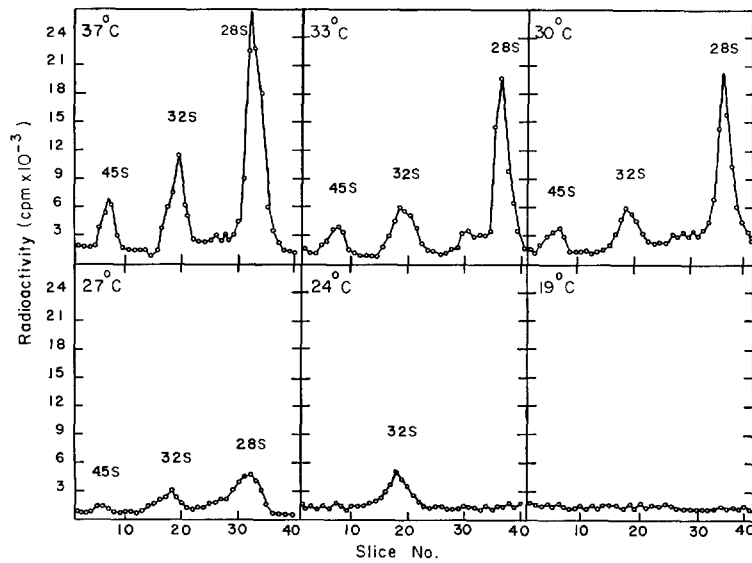


FIGURE 3 Synthesis and processing of 45S RNA. A culture of  $10^8$  cells was resuspended in 30 ml of medium and divided into six equal parts. Methionine- $^3\text{H}$  was added to a final concentration of  $50 \mu\text{Ci/ml}$ , and incubation was performed at the given temperatures for 3 hr. Electrophoresis was performed on 2.6% gels for 9 hr.

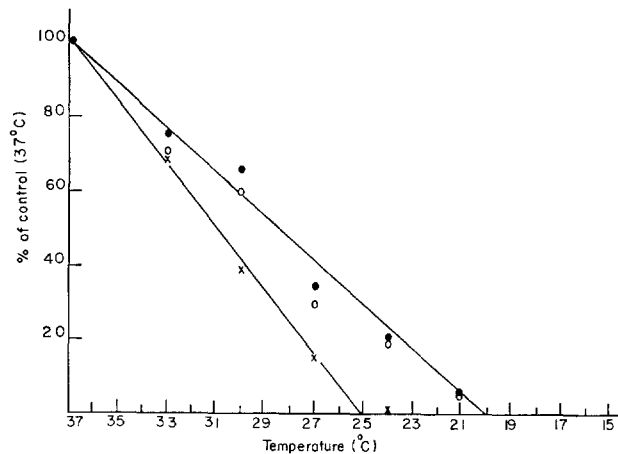


FIGURE 4 Synthesis as a function of the temperature of incubation. The counts per minute in the individual peaks of Fig. 3 were summed and plotted as the per cent of the  $37^\circ\text{C}$  control culture. Synthesis of 45S (○): sum of 45S, 32S, and 28S cpm and corrected for the estimated loss of the 18S cpm. Formation of 32S (●): sum of 32S and 28S RNA. Formation of 28S (×): 28S RNA cpm.

9 min in the presence of L-methyl- $^3\text{H}$ -methionine. During this period, labeled 45S RNA is formed; no label appears in 32S RNA. Equal portions of the culture were incubated for 1 hr at  $37^\circ$ ,  $33^\circ$ ,  $30^\circ$ ,  $27^\circ$ ,  $24^\circ$ ,  $19^\circ$ , and  $15^\circ\text{C}$ . As shown in Fig. 5, the processing of 45S RNA to 32S and 28S RNA decreases with temperature. 28S RNA formation

ceases between  $30^\circ$  and  $27^\circ\text{C}$  and 32S RNA accumulation is no longer detected between  $19^\circ$  and  $15^\circ\text{C}$ . When the counts in the 32S and 28S RNA peaks are summed for the different temperatures and plotted as the percentage of the control culture at  $37^\circ\text{C}$ , the curve in Fig. 6 is generated. In contrast with Fig. 4, the intersection of the line with

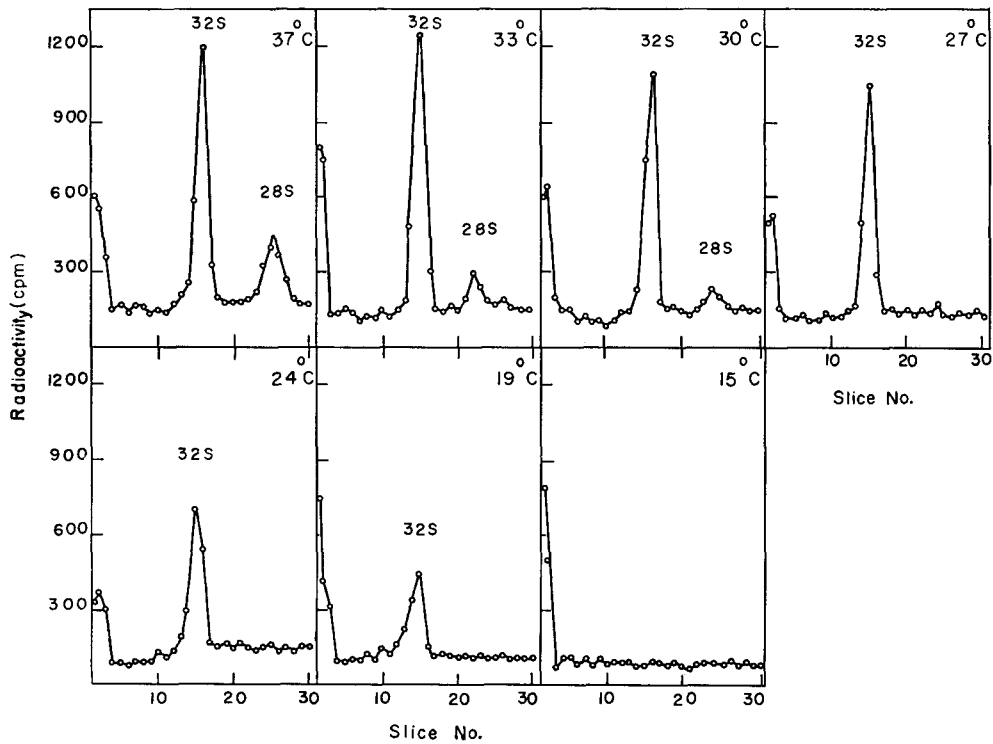


FIGURE 5 Processing of 45S to 32S RNA.  $6 \times 10^7$  cells were resuspended in 28 ml of medium containing  $50 \mu\text{Ci/ml}$  methionine- $^3\text{H}$ . The cells were incubated for 9 min at  $37^\circ\text{C}$ . The culture was divided into seven equal parts and the incubation was continued, at the temperatures indicated, for 1 hr before the isolation of the RNA and its electrophoresis on 2.9% gels for 7 hr.

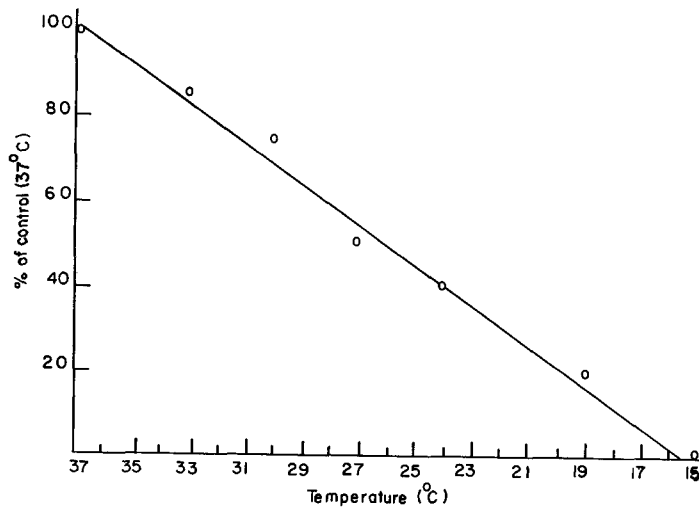


FIGURE 6 Processing of 45S to 32S RNA as a function of the temperature. From the data presented in Fig. 5, the counts in the 32S RNA peaks and in the 28S RNA peaks, when present, were summed for each temperature and plotted as the per cent of the control culture at  $37^\circ\text{C}$  versus the temperature of incubation.

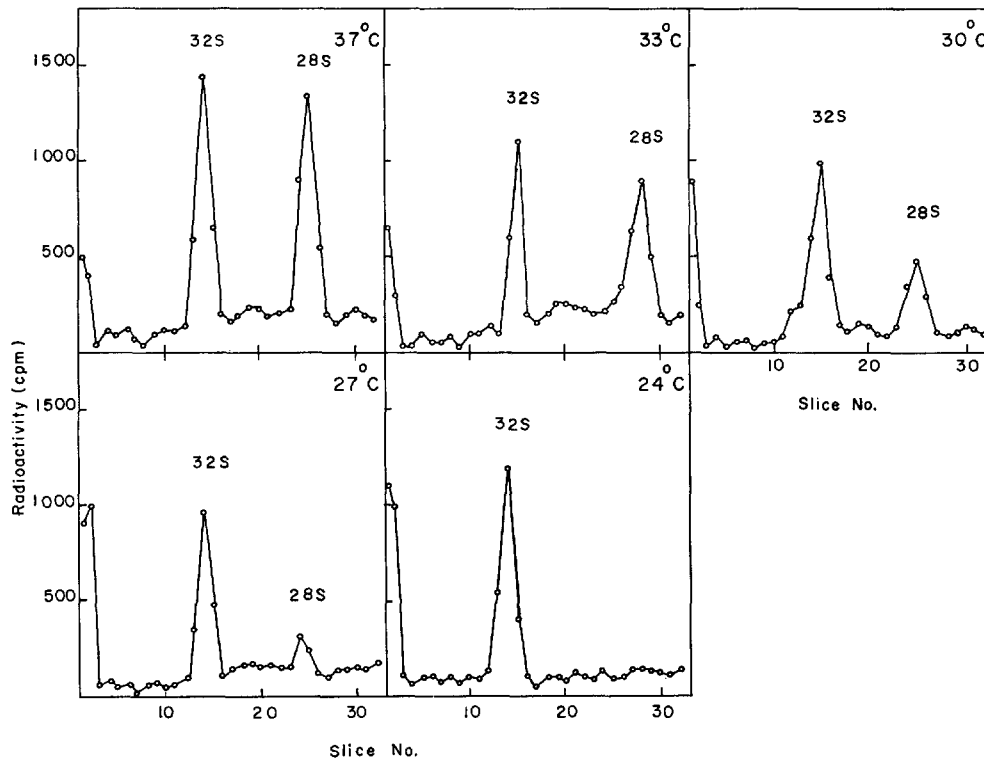


FIGURE 7 The processing of 32S to 28S RNA. A culture of  $10^8$  cells was resuspended in 20 ml of medium containing  $50 \mu\text{Ci/ml}$  of methionine- $^3\text{H}$ , and incubated for 30 min at  $37^\circ\text{C}$ . The culture was then divided into five equal parts and the incubation was continued for an additional 2 hr at the given temperature. Electrophoresis was performed on 2.9% gels for 7 hr.

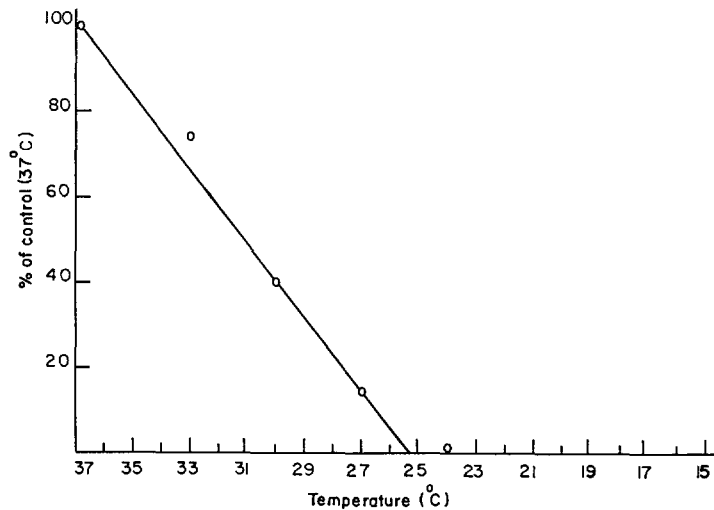


FIGURE 8 Processing of 32S to 28S RNA as a function of temperature. The counts per minute in the 28S RNA peaks in Fig. 7 were summed for each temperature and plotted as the per cent of the  $37^\circ\text{C}$  control culture *versus* the temperature of incubation.

the abscissa in Fig. 6 suggests that the cutoff temperature for the processing of 45S RNA to 32S RNA is near 15°C and not near 20°C as determined by continuous labeling.

#### The 32S RNA to 28S RNA Reaction

In a similar manner, the processing of 32S RNA was investigated. 32S RNA was labeled by incubation of the cells at 37°C for 30 min with L-methyl-<sup>3</sup>H-methionine. The culture was divided into equal portions which were incubated at 37°, 33°, 30°, 27°, and 24°C for an additional 2 hr. As shown in Fig. 7, the processing of 32S RNA was reduced with decreasing temperature until by 24°C there was no apparent accumulation of 28S RNA. When the counts in the 28S RNA peaks at the different temperatures are plotted as the percentage of the control counts at 37°C, the temperature at which the reaction is inhibited is near

25°C (Fig. 8). This temperature is in close agreement with that obtained during continuous labeling at reduced temperatures.

#### Effects of Prolonged Incubation on Intermediate Formation

Prolonged incubation (24 hr) of HeLa cells at reduced temperatures results in the accumulation of intermediates not observed during shorter incubations (3 hr). Cells which have been incubated for 24 hr in the presence of <sup>32</sup>PO<sub>4</sub> begin to accumulate 41S RNA at 33°C (Fig. 9). At 27°C this accumulation is greater than 45S RNA; there is also evidence for the accumulation of 36S RNA. At 23°C the identification of 45S RNA is complicated by a high background of RNA in that region of the gel. The main peak is 41S RNA with lesser amounts of 36S and 32S RNA. To reduce the level of background radioactivity in HnRNA, the

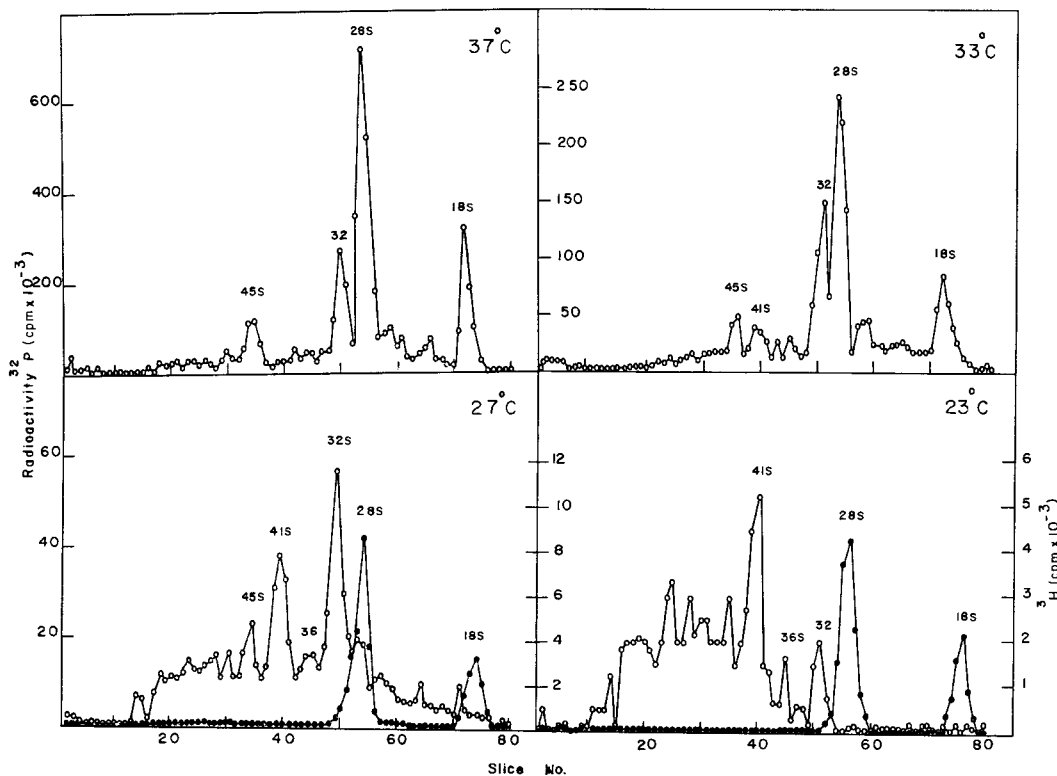


FIGURE 9 Ribosomal RNA synthesis during prolonged incubation at reduced temperatures.  $8 \times 10^7$  cells were resuspended in 20 ml of medium containing 500  $\mu\text{Ci/ml}$   $\text{H}_3^{23}\text{PO}_4$  and incubated for 24 hr at the indicated temperatures. The RNA was extracted and electrophoresed on 2.5% gels for 7 hr. Radioactivity,  $\circ$ ,  $^{32}\text{P}$ ;  $\bullet$ ,  $^3\text{H}$  RNA marker.



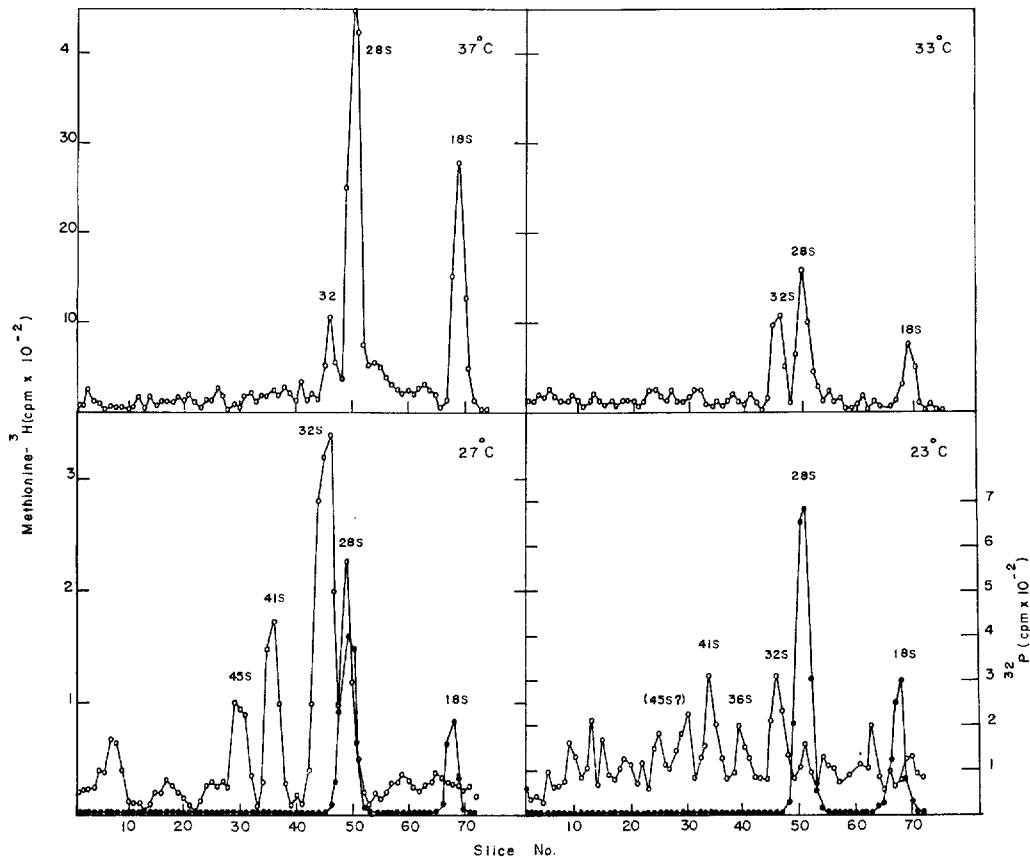


FIGURE 10 Methylation and processing of rRNA after prolonged incubation at reduced temperatures.  $2 \times 10^7$  cells were resuspended in 15 ml of medium containing  $20 \mu\text{Ci/ml}$  L-methyl- $^3\text{H}$ -methionine and incubated 18 hr at the indicated temperatures. The RNA was extracted and separated by electrophoresis on 2.6% acrylamide gels for 7 hr. Radioactivity:  $\circ$ ,  $^3\text{H}$ ;  $\bullet$ ,  $^{32}\text{P}$  RNA marker.

effect of prolonged temperature reduction was repeated with L-methyl- $^3\text{H}$ -methionine. Under these conditions, 41S RNA becomes more prominent at  $27^\circ\text{C}$ , while at  $23^\circ\text{C}$  the accumulation of 36S RNA becomes significant (Fig. 10). The identity of the RNA species was established by their mobility with respect to 28S and 18S RNA marker, using the logarithmic relationship between molecular weight and mobility which has been established for RNA analyzed on acrylamide gels (22). The necessity for marker RNA precluded the combination of  $^{32}\text{P}$  and L-methyl- $^3\text{H}$ -methionine incorporation in simultaneous label. The ratio of 45S:41S RNA during prolonged incubation is quite variable for all temperatures for reasons not fully understood. The time necessary for the accumulation of these intermediates precludes a closer examination of their formation and breakdown at this time.

#### *Integrity of Low Temperature Products*

The use of low-temperature incubation for studying the individual steps of RNA processing requires that the processing at suboptimal temperatures result in a normal final product. If the RNA molecules made at reduced temperature could be found to result in functional polyribosomes, then it could be assumed that the synthesis and processing at the reduced temperature produced normal ribosomal RNA. Accordingly, a cell population was incubated for 1.5 hr at  $27^\circ\text{C}$  in the presence of L-methyl- $^3\text{H}$ -methionine. A 1000-fold excess of nonradioactive methionine was added and the incubation was continued for an additional 3.5 hr at  $27^\circ\text{C}$  to allow the formation of 28S and 18S RNA molecules. The temperature was then raised to  $37^\circ\text{C}$  for 0.5 hr to allow the formation of polyribosomes. For the final minute of incubation

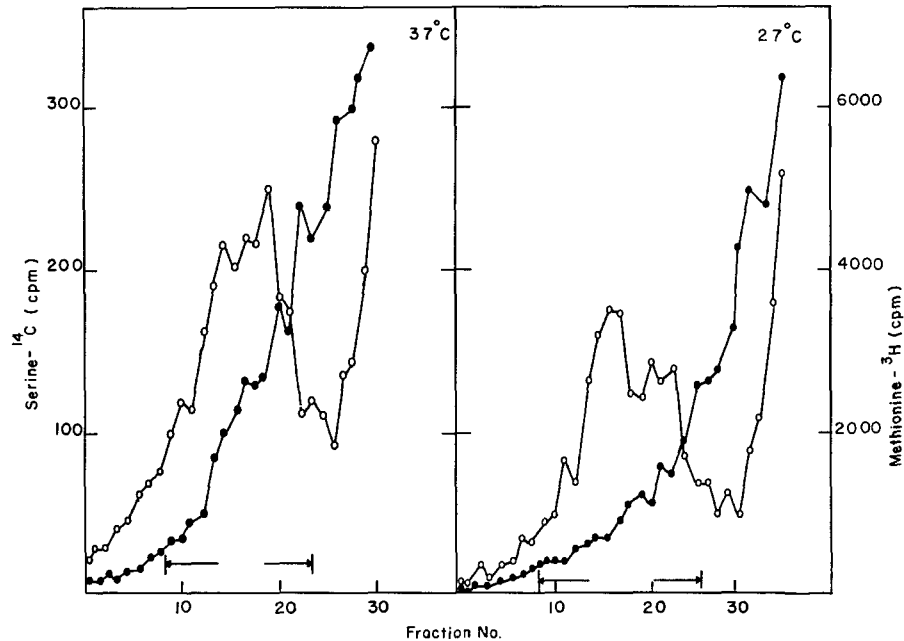


FIGURE 11 Polyribosomes containing ribosomal RNA formed at low temperature.  $8 \times 10^7$  cells were resuspended in 6 ml of medium containing 100  $\mu\text{Ci/ml}$  methionine- $^3\text{H}$ . The culture was divided into two equal parts which were incubated for 1 hr at 37° and 27°C, respectively. A 1000-fold excess of unlabeled methionine was added to each culture and the incubation was continued for an additional 3.5 hr. The temperature was then raised to 37°C for 29 min, when 10  $\mu\text{Ci}$  of serine- $^{14}\text{C}$  was added to each culture and the cultures were reincubated for an additional 1 min. The cells were chilled and a cytoplasmic extract was made as described in Materials and Methods. The extract was run on a 5–20% sucrose gradient for 1 hr at 20,000 rpm in an SW 25.2 rotor. Fractions (0.5 ml) were removed from the gradients and alternate fractions were made either 10% with TCA or 0.5% with SDS. The TCA fractions were Millipore filtered and assayed for radioactivity. The SDS fractions from the regions between the arrows were pooled for electrophoresis. Radioactivity:  $\circ$ ,  $^{14}\text{C}$ ,  $\bullet$ ,  $^3\text{H}$ .

serine- $^{14}\text{C}$  was added as an indicator of protein synthesis on the polyribosome (17). A similar culture maintained at 37°C was employed as a control. The nuclei and cytoplasm were separated and the cytoplasmic extract was layered upon a 5–20% sucrose gradient for polyribosome isolation. After centrifugation, alternate fractions were removed and made either 0.5% in SDS or 10% in trichloroacetic acid (TCA). The TCA-precipitated fractions were filtered onto Millipore filters, and the polyribosome region was considered to be that region showing serine- $^{14}\text{C}$  incorporation (Fig. 11). The SDS-treated fractions corresponding to the polyribosome region were pooled and the RNA was extracted and separated by electrophoresis. Fig. 12 shows that there is labeled 28S and 18S RNA isolated from the polyribosome region at both 37°C and 27°C, suggesting that the RNA made at 27°C is found associated with functioning

polyribosomes. Thus, the RNA made at 27°C appears normal by this criterion.

#### DISCUSSION

The experiments presented in this paper have demonstrated that the incubation of cells at suboptimal temperatures results not only in the reduced synthesis of ribosomal precursor RNA but in selective reduction of the subsequent processing of the precursor RNA. Evidence is further presented suggesting that the processing at reduced temperatures is normal in that the ribosomes formed find their way to polyribosomes actively engaged in protein synthesis.

When the incubation temperature is reduced from 37°C, the synthesis of 45S RNA exhibits a linear decrease, with no synthesis detectable below 19°C. This reduction is not caused by lack of available radioactive precursors, as the uptake and

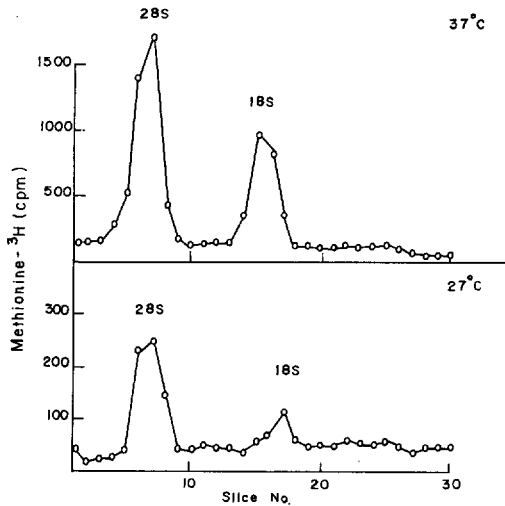


FIGURE 12 Ribosomal RNA from the polyribosome region. 2 vol of cold ethanol were added to the fractions from Fig. 11 made 0.5% with SDS. The RNA was concentrated and electrophoresed on 2.6% gels for 4 hr.

phosphorylation of uridine occurs at greater than 50% of the 37°C rate at 15°C. A similar observation has been extended to 4°C with chick embryo fibroblasts (23).

Examination of processing at lower temperatures of 45S RNA formed at 37°C revealed that 32S RNA is formed above 15°–17°C. Between 15°C and 25°C 32S RNA accumulates, and when a period of L-methyl-<sup>3</sup>H-methionine labeling is followed by an unlabeled methionine chase, it can be shown that in this temperature range 32S RNA is the only labeled intermediate accumulated.

With prolonged incubation at reduced temperatures 41S and possibly 36S RNA also accumulate, suggesting a situation different from short-term incubation where these intermediates are not accumulated. The effect of temperature on the formation and breakdown of these intermediates is difficult to measure at this time, due to the time needed for their accumulation; but as the 32S RNA is formed above 15°C, it would appear that these conversions are not highly temperature dependent.

Several other methods of accumulating intermediates of ribosomal RNA are known. When cells are starved for essential amino acids (24), or when protein synthesis is inhibited by cycloheximide or puromycin (25, 26), the rates of synthesis and processing of ribosomal RNA are modified. Like-

wise, infection with polio virus blocks normal formation of 28S RNA, allowing accumulation of the intermediates in the processing of 45S RNA (10). Low-temperature accumulation of intermediates has the advantage of being potentially less disruptive for the cell, and reversal can be achieved simply by raising the temperature.

The nature of the blocks in processing imposed by reduced temperatures is not known. Whether the enzymes needed for the processing are differentially sensitive to lower temperatures, or whether the energies of activation of the separate steps of the processing are significantly different from one step to another, cannot be discerned at this time. The fact, however, that 41S and 36S RNA accumulate only after prolonged exposure to low temperature suggests that for the accumulation of these RNA species there is inhibition or elimination of some components necessary for further processing.

As interesting as is the general picture of the synthesis and processing of ribosomal precursor at temperatures below 37°C, the value of the approach lies in the potential it provides for the *in vivo* accumulation of intermediates and for studying more precisely the individual steps of the processing.

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