KINETICS OF INCORPORATION OF URIDINE-C^{14} INTO L CELL RNA

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ABSTRACT Five components have been isolated from L cells by a combination of phenol extraction procedures and sedimentation analysis through sucrose gradients. These components are identified by their sedimentation rates. The 50S and 40S components are derived from the nucleus, the 32S and 18S from ribosomal RNA, and the 4S fraction is the soluble RNA of the cell. L cells were supplied with uridine-C^{14} under steady-state conditions and the rate of uptake of C^{14} into each component was measured. Analysis of the results suggests that the delay in entry of C^{14} into ribosomal RNA is occasioned by two sequential precursors and that 50S and 40S RNA meet the kinetic requirements for these precursors. 4S RNA seems to contain two components that label at different rates.

INTRODUCTION

When mammalian cells in culture are exposed to labeled RNA precursors, RNA of the nucleus is the first fraction of cellular RNA to incorporate the label. After a lag, label then begins to appear in cytoplasmic RNA. The obvious possibility that nuclear RNA is a precursor of cytoplasmic RNA has been the subject of a considerable amount of work and some controversy over a period of years (for review of the literature see Graham and Rake, 1963).

Resolution of this controversy depends, in part, upon methods for separating the various components of cellular RNA. The rapidly labeled nuclear RNA can be obtained either from isolated nuclei or from whole cells by phenol extraction procedures (Hiatt, 1962; Scherrer and Darnell, 1962; Perry, 1962; Cheng, 1962; Monier, 1962; Harris and Watts, 1962; Harris *et al.*, 1963; Zimmerman *et al.*, 1963; Homma and Graham, 1963). There is relatively very little of this material in the nucleus and it can be readily detected only by its content of label. It has generally been found to be more or less polydisperse by sedimentation analysis in sucrose gradients, but Scherrer and Darnell (1962) have shown recently that, under carefully controlled conditions, two fairly well resolved components sedimenting at rates of approximately 50S and 40S can be obtained. Phenol extraction of whole cells also removes three other RNA components that are observed in sucrose gradient analyses to sediment at rates of 30 to 32S, at 18S, and at about

4S (Hiatt, 1962; Scherrer and Darnell, 1962; Perry, 1962; Homma and Graham, 1963; Cheng, 1962; Monier, 1962). The 32S and 18S components are constituents of ribosomal RNA and very largely of cytoplasmic origin while the 4S material contains the amino acid transfer RNA of the cell (Harshaw *et al.*, 1962).

The 50S component is the most rapidly labeled RNA of the cell, followed closely by the 40S fraction (Scherrer and Darnell, 1962). Recently, Perry (1962) and Scherrer *et al.* (1963) have suggested that these nuclear components are largely incorporated into ribosomal RNA without breaking down, en route, into the acid-soluble precursor pools of the cell. On the other hand, Harris and his associates (Harris and Watts, 1962; Harris *et al.*, 1963; Harris, 1963) have maintained that the rapidly labeled nuclear RNA breaks down after its synthesis and that ribosomal RNA is formed independently of the nuclear components.

In the present work we have attempted to get some further information on possible precursor-product relationships by determining the rates of labeling of the several cellular RNA components under essentially "steady-state" conditions (Graham and Rake, 1963). L cells, growing exponentially in suspension, were exposed to uridine-C^{14} in such a manner that the precursor was assimilated at a maximum rate throughout the experiment. Two phenol extraction procedures and sucrose gradient sedimentation analyses were employed to separate the 50S and 40S nuclear RNAs from the 32S and 18S ribosomal RNA and from 4S RNA. An analysis of the kinetics of labeling of each component is presented.

MATERIALS AND METHODS

1. *Growth and Labeling of Cells*

Strain L mouse cells were grown in medium CMRL-1066 (Siminovitch *et al.*, 1957), from which the liver coenzyme concentrate and nucleotides were omitted, supplemented with 10 per cent horse serum. In many of the later experiments Eagle's basal mixture (Eagle, 1959) in Earle's salt solution with 10 per cent horse serum was used as the medium. Similar results were obtained in the labeling experiments with these media. Cell cultures were stirred magnetically in Erlenmeyer flasks with the aid of teflon-covered magnetic bars lying loosely in the flasks. At all times cell concentrations were maintained between 1 to 6 $\times$ 10^5 cells per ml by daily dilution with fresh medium. Only those cultures that had maintained logarithmic growth, with a generation time of 24 hours, for the preceding several generations were used for experiments.

During the labeling of all cultures the concentrations of exogenous uridine-C^{14} were kept above the "saturating concentration" to ensure that the cells took up the precursor at a maximum rate (Rake and Graham, 1962).

2. *Isolation of RNA*

Two different methods involving extractions with phenol were used to isolate RNA from cell cultures.

(*a*) *Phenol-SDS Procedure.* This method was used essentially as described by Scherrer and Darnell (1962). The cell culture was centrifuged at room temperature

and the sediment was immediately frozen at −70° and maintained at this temperature until required. To the frozen sediment were added 4 ml of a solution containing 0.14 M LiCl, 10^{-2} M acetate buffer, pH 5.0, 10^{-3} M Mg^{++}, 4×10^{-4} per cent polyvinyl sulfate, 0.5 per cent sodium dodecyl sulfate (SDS), and 4 ml of water-saturated phenol. The mixture was vigorously shaken by hand for several seconds. This procedure released considerable amounts of cellular DNA whose viscosity made it difficult to perform further manipulations. DNA was removed by heating the mixture to 60° for 3 minutes and quickly cooling it to 4°. The two phases were separated by centrifuging the mixture at low speed and the aqueous phase, containing the RNA, was removed. Phenol was extracted from the aqueous phase by shaking it with ether, and the residual ether was removed in a stream of nitrogen. Six volumes of ethanol were then added at 4° to precipitate the RNA which was centrifuged and redissolved in a salt solution containing 10^{-2} M tris, pH 7.6, 0.14 M LiCl, 10^{-3} M Mg^{++} (TLM buffer).

(*b*) *Phenol-EDTA Procedure.* Cultures were sedimented and the pellet of cells frozen as in the previous method. The frozen pellet was taken up in 2 ml of TLM buffer containing 4×10^{-4} per cent polyvinyl sulfate, 0.1 per cent ethylenediaminetetraacetate (EDTA), and 2 ml of water-saturated phenol. The mixture was shaken vigorously, the two phases were separated by sedimentation, and phenol was removed from the aqueous phase as before. RNA was precipitated, centrifuged, and then redissolved in TLM buffer as with the phenol-SDS method.

3. *Sephadex Column Fractionation*

Prior to sedimentation analysis in sucrose gradients, all specimens of RNA (except as noted for one specific experiment) were purified by passage through a column of sephadex G-25 gel, 2.5 cm in diameter × 20 cm, to remove low molecular weight components. The column was equilibrated with TLM buffer before addition of the sample and afterwards developed with the same buffer. Similar fractionation of RNA on sephadex columns has been described in detail (Homma and Graham, 1963). Only that fraction of RNA that flowed through the column in the outside volume, V_o, was utilized for subsequent analysis.

4. *Sucrose Gradient Sedimentation Analysis*

After passage through a sephadex column RNA samples were precipitated with ethanol, redissolved in a small volume of TLM buffer, and 0.3 ml was layered over 4.5 ml of a linear sucrose gradient (Britten and Roberts, 1960). The steepness of the gradient was varied from 5 to 20 per cent to 5 to 40 per cent sucrose from one experiment to another depending on the circumstances. Sucrose was dissolved in TLM buffer for preparation of the gradients. Centrifugation was generally for 3 hours at 35,000 RPM in the SW39 head of the Spinco model L centrifuge. The bottom of the tube was then punctured and consecutive samples were collected dropwise into 0.5 ml of distilled water.

5. *Assay of* C^{14}

All samples of C^{14} were assayed with a liquid scintillation spectrometer (Tracerlab or tri-carb) using the liquid scintillation system of Bray (1960).

GENERAL PROCEDURE

1. *Efficiency of the Extraction Procedures.* The following experiment was performed to determine the efficiency of the two phenol extraction procedures in isolating

RNA from L cells. A culture of L cells was labeled for 30 minutes with uridine-C^{14}. Three samples, each containing 10^8 cells, were removed from the culture and the cells sedimented. The first pellet of cells was extracted with 5 per cent perchloric acid at 4° and the insoluble material centrifuged. All the nucleic acid of this sediment was then extracted by heating it at 95° for 10 minutes with 5 per cent perchloric acid. RNA in the extract was determined by the orcinol method (Mejbaum, 1939) and DNA was determined by the diphenylamine reaction (Burton, 1956). The results of the colorimetric and C^{14} assays are shown in Table I. The remaining two pellets of cells from the culture

TABLE I

COMPARISON OF THREE EXTRACTION METHODS USED TO REMOVE NUCLEIC ACID COMPONENTS FROM C^{14}-LABELED L CELLS*

Fraction	Aqueous extract prepared by		
	Phenol-SDS	Phenol-EDTA	Whole cells
C^{14} insoluble in 5 per cent perchloric acid, CPM	43,100	4,440	43,500
Total RNA insoluble in 5 per cent perchloric acid, as μmoles of adenosine‡	3.04	1.84	3.00
Total DNA insoluble in 5 per cent perchloric acid, as μmoles of deoxyadenosine‡	0	0	1.11

*Each extraction procedure was carried out on a sample, containing 10^8 cells, taken from a culture that had been labeled with uridine-C^{14} for 30 minutes.
‡Orcinol and diphenylamine reactions were carried out on the material extracted from the cold perchloric acid precipitate with hot perchloric acid.

were extracted by one or the other of the two phenol procedures and the results of the various assays are also shown in Table I.

The essential results are as follows: (*a*) neither phenol precedure removed an appreciable amount of DNA; (*b*) the phenol-SDS procedure isolated the entire acid-insoluble RNA of the cell, determined either by C^{14} or by orcinol assay (see also, Scherrer *et al.*, 1963); (*c*) by colorimetric analysis the phenol-EDTA method removed about 60 per cent of the cellular RNA (see also Rake and Graham, 1962). Only about 10 per cent of the labeled RNA was isolated by this method, however. As will be shown later, the phenol-EDTA procedure selectively extracts certain RNAs from the cell.

2. *Sedimentation Analysis of RNA Extracted by the Phenol-SDS Method.* Fig. 1*a* shows the sedimentation analysis of RNA that had been extracted from cells labeled for 30 minutes with uridine-C^{14} and then passed through a sephadex column. Three major components are revealed by optical density measurements. The 32S and 18S components are derived from ribosomal RNA and the 4S material contains the soluble RNA of the cell (Hiatt, 1962). Sedimentation values were determined in the analytical ultracentrifuge

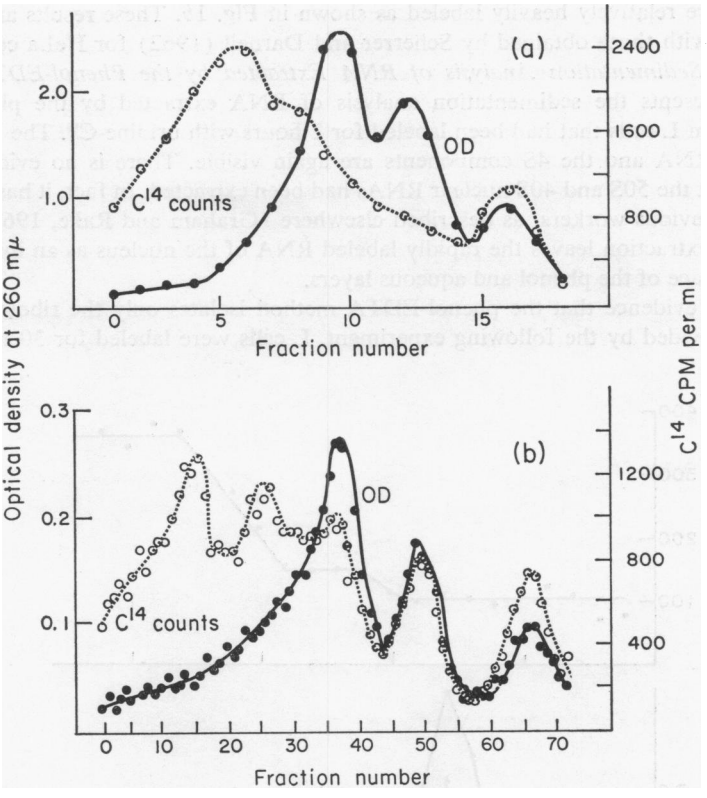

FIGURE 1 Sucrose gradient sedimentation analysis of RNA extracted from L cells by the phenol-SDS procedure. (*a*) Cells were labeled for 30 minutes with uridine-C^{14}: 4-drop samples collected from the centrifuge tube. (*b*) Cells were labeled for 1.5 hours and 1-drop samples collected. In routine sedimentation analyses, 3- or 4-drop samples were generally collected after centrifugation. 1-drop samples were taken in the experiment of Fig. 1*b* to demonstrate the maximum resolution that could be obtained between the 50S, 40S, and 32S components by this technique. Control experiments showed that all of the C^{14} in each of the fractions could be precipitated with 5 per cent perchloric acid and that digestion with ribonuclease converted all the C^{14} to an acid-soluble form.

equipped with ultraviolet optics, through the kindness of Dr. R. A. Brown. The C^{14} profile in Fig. 1*a* does not correspond to the optical density but shows a fourth peak sedimenting at the rate of approximately 50S, and a fifth at 40S that appeared as a shoulder on the fastest moving component. These two latter components are derived from the cell nucleus. Their sedimentation rates were determined by plotting the three known values, 32S, 18S, and 4S, against their positions in the gradient and extrapolating the resulting straight line. (They are of more use in identifying the components for further reference than for any relevance they may have to true sedimentation rates.) When the cells were labeled for longer periods C^{14} began to enter the 32S and 18S components and they became progressively more heavily labeled. Even after a 1.5 hour period these ribosomal RNA com-

ponents were relatively heavily labeled as shown in Fig. 1*b*. These results are in general agreement with those obtained by Scherrer and Darnell (1962) for HeLa cells.

3. *Sedimentation Analysis of RNA Extracted by the Phenol-EDTA Method.* Fig. 2 represents the sedimentation analysis of RNA extracted by the phenol-EDTA method from L cells that had been labeled for 2 hours with uridine-C^{14}. The 32S and 18S ribosomal RNA and the 4S components are again visible. There is no evidence in this analysis that the 50S and 40S nuclear RNAs had been extracted. In fact, it has been found by many previous workers, as described elsewhere (Graham and Rake, 1963), that this method of extraction leaves the rapidly labeled RNA of the nucleus as an insoluble mass at the interface of the phenol and aqueous layers.

Further evidence that the phenol-EDTA method isolates only the ribosomal and 4S RNA is provided by the following experiment. L cells were labeled for 30 minutes with

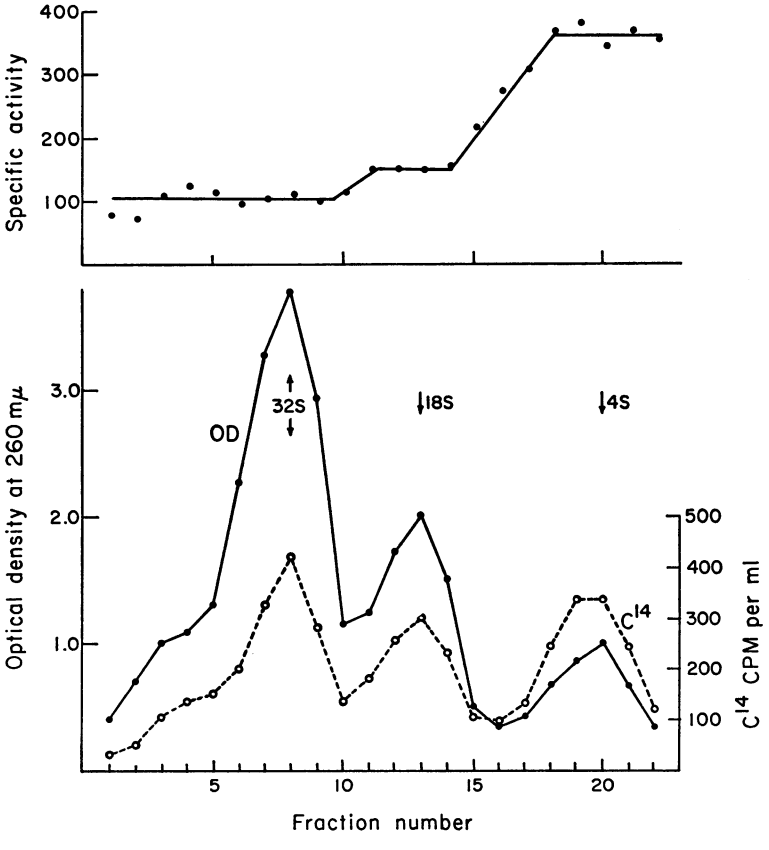

FIGURE 2 Sucrose gradient sedimentation analysis of RNA extracted from L cells by the phenol-EDTA procedure. The cells had been labeled for 2 hours with uridine-C^{14}. Specific activities shown in the upper panel were calculated as CPM per optical density unit. Control experiments showed that all the C^{14} in each of the fractions could be precipitated with 5 per cent perchloric acid in the cold and that digestion with ribonuclease converted all of the C^{14} to an acid-soluble form.

uridine-C^{14} and then extracted by the phenol-EDTA procedure. The aqueous phase was carefully removed and the RNA precipitated with ethanol and analyzed by sucrose gradient sedimentation (Fig. 3*a*). The phenol phase and attendant interfacial precipitate resulting from the first extraction were then shaken at 60° with a solution containing 0.14 M LiCl,10^{-3} M acetate buffer, pH 5.0, 10^{-3} M Mg^{++}, 4×10^{-4} per cent polyvinyl sulfate, 0.5 per cent SDS. The aqueous phase was separated and the phenol removed by extraction with ether. After the removal of the ether with a stream of nitrogen at 4°, a sample of the solution was analysed by sedimentation through a sucrose gradient. The results are shown in Fig. 3*b*. In this particular experiment the RNA was not passed through a column of sephadex gel before sedimentation analysis. Much of the C^{14} in the 4S RNA fractions is, therefore, the result of labeled pool components carried through the isolation procedure. However, it is clear from Fig. 3*a* that there was an insignificant amount of label

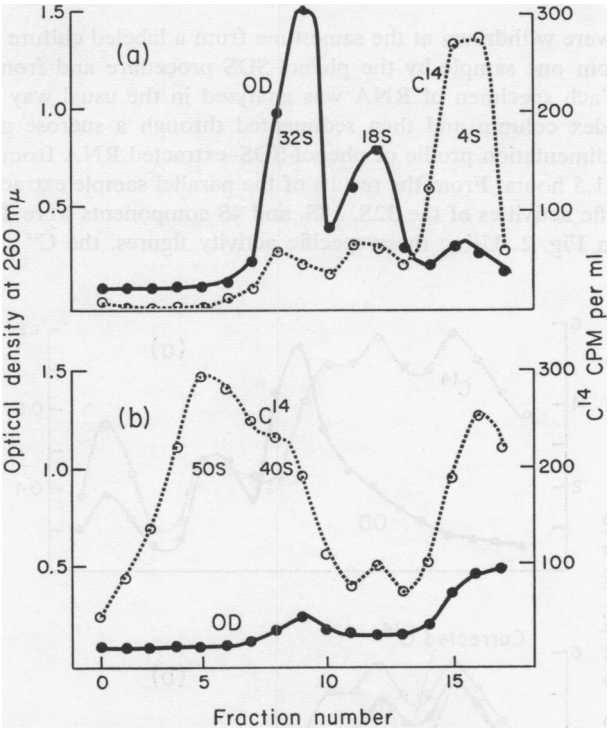

FIGURE 3 (*a*) Sedimentation analysis of RNA extracted from L cells by the phenol-EDTA method. The cells had been labeled for 30 minutes with uridine-C^{14}. (*b*) Sedimentation analysis of RNA extracted by the phenol-SDS method from the isoluble residue remaining after the phenol-EDTA extraction.

associated with the 32S and 18S components and that none of the rapidly labeled nuclear RNA had been isolated. Subsequent phenol extraction of cellular debris in the presence of SDS separated this nuclear RNA as the sedimentation pattern of Fig. 3*b* shows. A small amount of optically dense material in fraction nine is the 32S ribosomal RNA

component. It should be noted that the total amount of nuclear RNA-C^{14} isolated in this second extraction varied from one experiment to another.

The results of the last two sections and of Table I indicate, therefore, that the phenol-SDS extraction procedure isolates the total RNA of the cell, in agreement with the results of previous workers, and that the phenol-EDTA method removes only the ribosomal and *s*-RNA (soluble RNA, 4S RNA), perhaps incompletely.

4. *Incorporation of* C^{14} *into the Nuclear RNA Components.* As was first shown by Scherrer and Darnell (1962) for HeLa cells, and is seen in Fig. 1 of this paper, the 50S nuclear RNA became labeled at a much faster rate than the 40S component in short term experiments. With more prolonged periods of labeling, C^{14} began to enter the ribosomal RNA (32S and 18S) and further incorporation of C^{14} into the two nuclear components was then obscured. In order to determine the labeling kinetics of the nuclear RNAs, results from the two phenol extraction procedures were combined in the following way.

Two samples were withdrawn at the same time from a labeled culture of L cells. RNA was extracted from one sample by the phenol-SDS procedure and from the other with phenol-EDTA. Each specimen of RNA was analysed in the usual way by being passed through a sephadex column and then sedimented through a sucrose gradient. Fig. 4*a* represents the sedimentation profile of phenol-SDS–extracted RNA from L cells that had been labeled for 1.5 hours. From the results of the parallel sample extracted with phenol-EDTA, the specific activities of the 32S, 18S, and 4S components were determined in the manner shown in Fig. 2. Using these specific activity figures, the C^{14} profile of Fig. 4*a*

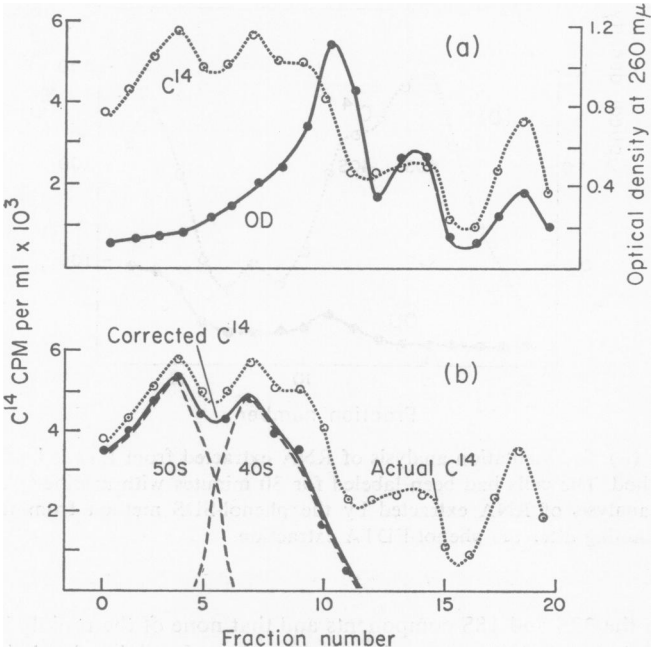

FIGURE 4 Method for determining the C^{14} content of the 50S and 40S components of nuclear RNA.

was then corrected for the contribution made by the 32S, 18S, and 4S components. The "actual" and "corrected" C^{14} profiles are shown in Fig. 4*b*. To evaluate the amount of C^{14} incorporated into each of the nuclear components, curves were fitted under the 50S and 40S peaks, as shown in Fig. 4*b*. The total area under each of these curves was then integrated with a planimeter.

There is obviously room for error in this type of analysis. The 50S and 40S components are not particularly well resolved from each other or from the ribosomal RNA. Further, as the cells were labeled for longer periods, the amount of C^{14} taken up by the ribosomal RNA became vastly greater than that in the nuclear components and the correction became large. Nevertheless, this general method was employed to estimate the kinetics of labeling of the two nuclear components as described in the next section.

RESULTS

1. *Kinetics of Labeling of the Cellular RNA Components.* The phenol-EDTA isolation procedure was used in determining the labeling of 32S, 18S, and 4S RNA since it isolated these components free of any of the rapidly labeled RNA of the nucleus. Uridine-C^{14} (1.25×10^5 CPM per μmole) was added at a concentration of 4.4×10^{-5} μg per cell to a large, exponentially growing culture containing 3.4×10^5 cells per ml. This concentration of uridine is sufficiently high to ensure that it will be taken up by the cells at a maximum rate over a period of 24 hours (Rake and Graham, 1962). Growth of the culture was continued and samples containing approximately 1.5×10^8 cells were removed at intervals. RNA was extracted from each sample, passed through a sephadex column, and analyzed by sedimentation through a sucrose gradient. The specific activity of each component was determined by the procedure indicated in Fig. 2 and the results are shown in Fig. 5.

During such experiments it was necessary to know whether there was any change in the specific activity of exogenous uridine-C^{14}. This point was checked in the following way. Samples of culture were taken at time zero, after the addition of uridine-C^{14}, and at various later intervals. After centrifuging out the cells, perchloric acid was added to the supernatant solution at 4° to a concentration of 10 per cent. The acid-soluble material was evaporated, hydrolyzed with concentrated perchloric acid, and the uracil isolated by paper chromatography. No change in specific activity of uracil was observed during several generations of cellular multiplication, and no cytosine-C^{14} was found on the chromatograms.

The kinetics of labeling of the 50S and 40S nuclear components were determined as follows. A large culture of L cells was labeled under conditions similar to those just described. At intervals two samples were withdrawn, the RNA was extracted by one or the other of the two phenol methods and the total amounts of label in each of the 50S and 40S components were determined by the method described in Section 4 of General Procedure. The results are shown in Fig. 6.

In order for the results of different experiments to be directly comparable it is

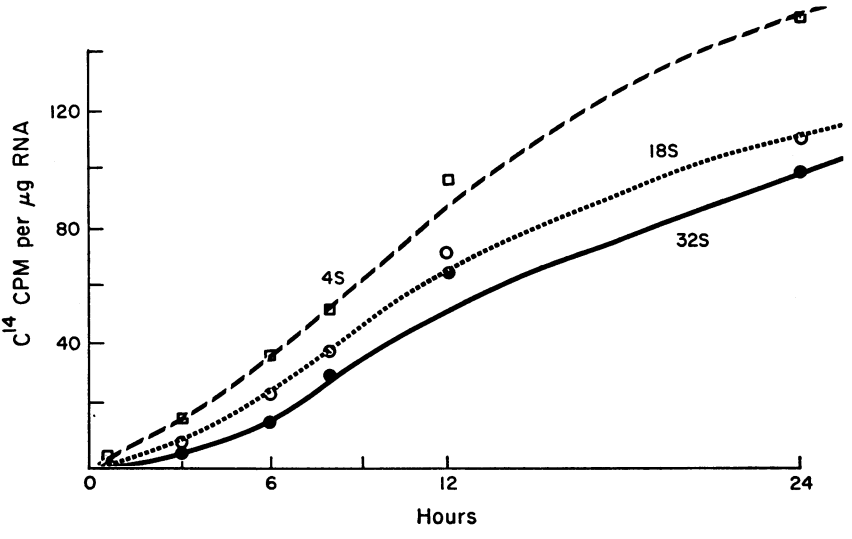

FIGURE 5 Kinetics of incorporation of exogenously supplied uridine-C^{14} into the 32S, 18S, and 4S components of L cell RNA.

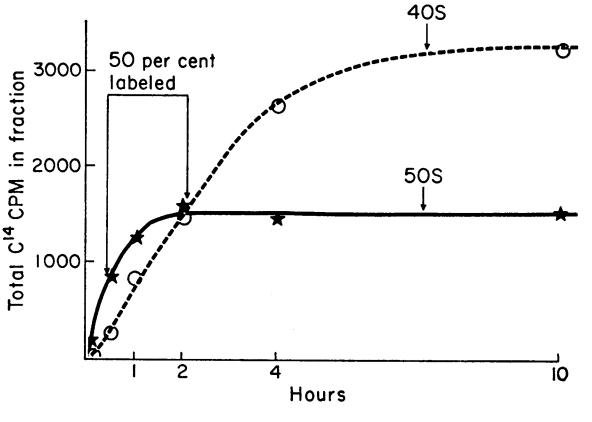

FIGURE 6 Kinetics of incorporation of exogenously supplied uridine-C^{14} into the 50S and 40S components of L cell RNA.

necessary to refer the rates of labeling to some known parameters of the system, namely, the specific activity of the exogenous precursor and the generation time of the culture. It is known from previous work (Rake and Graham, 1962) that the uracil and cytosine of ribosomal RNA reach 48 per cent of the specific activity of the medium in one generation. The 32S component, shown in Fig. 5, was therefore assumed to be 48 per cent labeled in 24 hours. Using this estimate, all the results of Fig. 5 and of another similar experiment were calculated as percentages

of the maximum attainable amount of label and are plotted in Fig. 7. Both the 50S and 40S components are fully labeled by 10 hours (Fig. 6). Since it has been shown that the *de novo* syntheses of uracil and cytosine are completely suppressed under the conditions of the present experiments (Rake and Graham, 1962), both components have reached the specific activity of the medium when they are fully labeled. For each component, the amount of C^{14} assimilated at any time was calculated as a percentage of the maximum and plotted in Fig. 7.

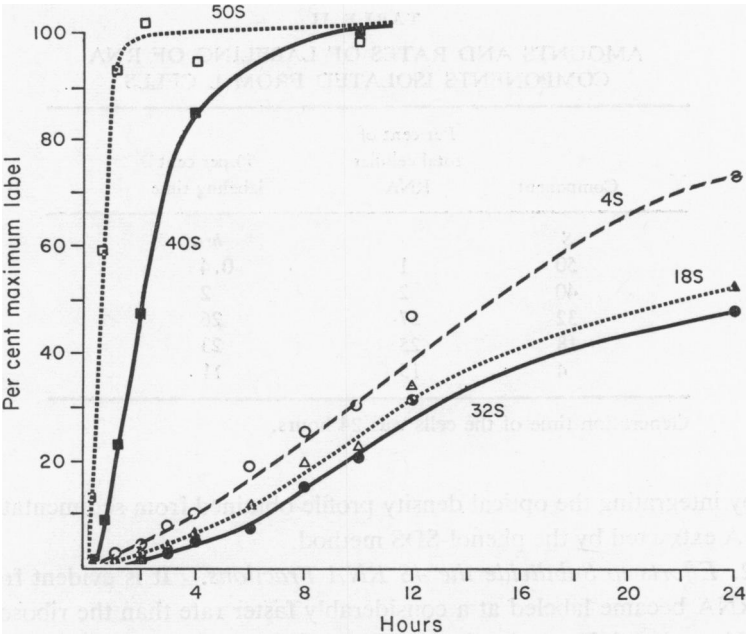

FIGURE 7 Kinetics of incorporation of uridine-C^{14} into the 50S, 40S, 32S, 18S, and 4S components.

The 50S component was almost fully labeled in 1 hour, while it took 6 to 10 hours for the 40S material to achieve maximum labeling. There was a considerable delay in the entry of label into the 40S component and an even greater lag before it entered ribosomal RNA. This sequence is similar to that observed for HeLa cells (Scherrer and Darnell, 1962; Scherrer *et al.*, 1963). It might be mentioned also that Perry *et al.* (1961), using autoradiographic techniques on HeLa cells labeled with cytidine-H^3, observed the nuclear RNA to be almost fully labeled in about 6 hours. In fact, the kinetics of labeling of the 50S and 40S components (Fig. 6) correspond quite closely to the labeling of the nuclear (non-nucleolar part) and the nucleolar RNAs, respectively, described by Perry *et al.* (1961). A more extended discussion of the curves of Fig. 7 will be presented later.

An estimate can be made of the quantity of each of the 50S and 40S components in the cell from the amount of C^{14} they contain when fully labeled at 10 hours. This calculation was made utilizing the known specific activity of the exogenous precursor and the assumption that both components contain 50 per cent of their nucleotides as pyrimidine nucleotide. The results are shown in Table II with the time required to become 50 per cent labeled. Also included in the table are estimates of the relative amounts of 32S, 18S, and 4S RNA. These latter figures were

TABLE II

AMOUNTS AND RATES OF LABELING OF RNA COMPONENTS ISOLATED FROM L CELLS

Component	Per cent of total cellular RNA	50 per cent labeling time
S		*hrs.*
50	1	0.4
40	2	2
32	57	26
18	25	23
4	15	11

Generation time of the cells was 24 hours.

derived by integrating the optical density profile obtained from sedimentation analysis of RNA extracted by the phenol-SDS method.

2. *Efforts to Subdivide the 4S RNA Fractions.* It is evident from Fig. 7 that 4S RNA became labeled at a considerably faster rate than the ribosomal components. If any breakdown of cellular RNA occurred during the isolation procedure, the products would probably appear in the 4S fraction and thereby distort its labeling kinetics. As one test of this possibility 4S RNA was separated in three different ways from a common culture. L cells were labeled for 24 hours with uridine-C^{14}, the RNA was extracted by the phenol-EDTA procedure, and divided into three parts. In method 1, the RNA was passed through a sephadex column and the 4S RNA component was then isolated after sedimentation in a sucrose gradient. In method 2, the 32S and 18S RNA components were precipitated with streptomycin (Harshaw *et al.*, 1962; Rake and Graham, 1962), discarded, and six volumes of ethanol were added to the supernatant solution. The 4S RNA material thus precipitated was dissolved in TLM buffer, passed through a sephadex column, and isolated after sedimentation through a sucrose gradient. With method 3, 4S RNA was prepared according to the procedure of Harshaw *et al.* (1962); 32S and 18S RNA were precipitated with streptomycin and discarded. The 4S RNA was precipitated by the addition of two volumes of ethanol to the supernatant solution and redissolved

in TLM buffer. 4S RNA obtained by the three methods had the same specific activity. These methods are sufficiently different that any gross contamination of the 4S RNA by breakdown products of ribosomal RNA or of the rapidly labeled nuclear RNA should have been apparent. As an additional point of interest, 4S RNA isolated after extraction of labeled cells by the phenol-SDS procedure had the same labeling kinetics as shown for the 4S fraction in Fig. 7.

In any event, it is clear that contamination of the 4S fraction by breakdown of ribosomal RNA could not *increase* the specific activity of the fraction. Further, as has been shown in Fig. 7, the nuclear components are almost completely labeled by 4 hours. If degradation of the 50S and 40S components was contributing labeled material to 4S RNA, the contamination should be apparent during the first 4 hours or so by a rapid rise in specific activity of the fraction. Such contamination should have a negligible influence on the specific activity of 4S RNA at later times, since the total amount of label in the nuclear RNA then becomes relatively very small. Thus, the shape of the kinetic curve for 4S RNA in Fig. 7 and the rate of labeling cannot be explained on the grounds that the fraction contains breakdown products of other RNAs.

It has been suggested that s-RNA isolated by phenol extraction and precipitated with ethanol may contain considerable amounts of nucleoside mono- and polyphosphates that are coprecipitated and difficult to remove by simple dialysis (Sluyser and Bosch, 1962). Such coprecipitated nucleoside phosphates can, however, be removed by prolonged dialysis against salt solution (Bosch *et al.*, 1961). In the present work, the specific activity of labeled s-RNA was not changed by dialysis against 1 M NaCl for several days. These results suggest that the rate of labeling of 4S RNA is not due to contamination with other cellular components.

DISCUSSION

Inspection of the curves of Fig. 7 immediately suggests that the 50S and 40S RNAs might constitute a sequence of precursors of ribosomal RNA. However, such kinetics are notoriously open to alternative interpretations and something more than a qualitative assessment of the data is necessary. To examine in more detail the possibility of such a sequential relationship we have analyzed these results by the mathematical procedures of Britten and McCarthy (1962) and McCarthy *et al.* (1962). The symbols and equations used here are those of Britten and McCarthy and reference should be made to their paper for a thorough discussion of the mathematical technique.

In Fig. 8 are plotted values of a function, ϕ, representing the newly synthesized fraction of a component, for each of the 50S, 40S, and 32S RNAs (ϕ_{50}, ϕ_{40}, ϕ_{32}) against corresponding values of τ. ϕ in each case is the product of two measured values, the fraction of the total cellular RNA represented by the component (Table II) and the fraction of its total attainable amount of label (Fig. 7) at any given

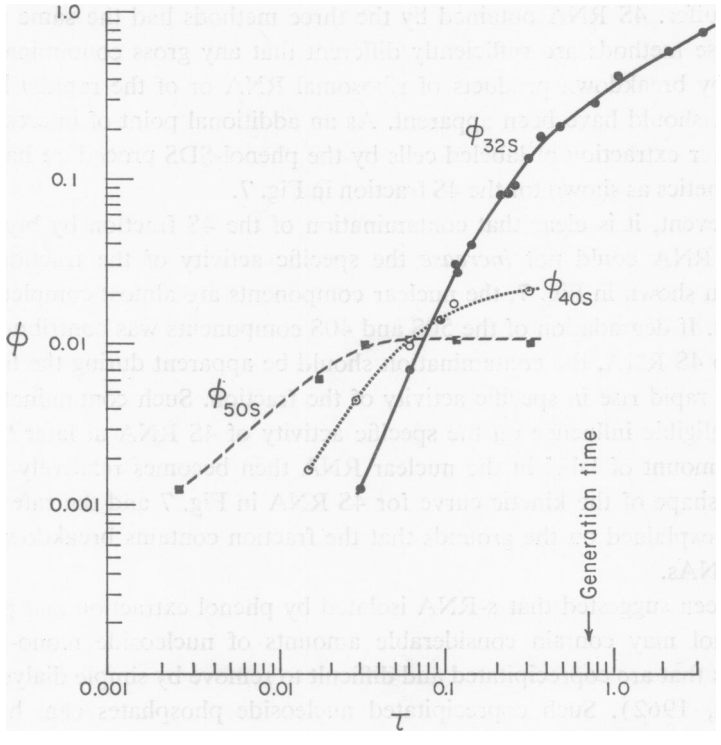

FIGURE 8 Log-log plot of the fraction of newly synthesized RNA (ϕ) present in the 50S, 40S, and 32S components as a function of time. ϕ was determined as described in the text. The time, τ, is defined by the equation for growth $Q = Q_0 e^{\tau}$.

time. Values of τ are obtained from the expression $Q = Q_0 e^{\tau}$ which represents the growth rate of the culture or of any cellular component.

The most extensive and dependable data in Fig. 8 are those for the 32S component, ϕ_{32}. This curve includes the results from two experiments performed during the present work and the uracil specific activity data for ribosomal RNA obtained from the previous paper (Rake and Graham, 1962, Fig. 4). In general, ϕ has the property that, at early times after addition of label to the culture, the function should approach linearity. It has the further property that the slope of this straight line should indicate the number of sequential precursor steps by which the label is delayed before its entry into the component. In the case of ϕ_{32}, the slope of the curve at early times, approximately τ^3, is equivalent to a delay by two sequential precursors. ϕ_{50} and ϕ_{40} answer the requirements for these precursors since they have initial slopes of τ and τ^2, respectively. The initial slope, τ, for ϕ_{50} means that this component is synthesized directly from the nucleotide pools and further suggests that there must be virtually no delay in the equilibration of its nucleotide precursors with exogenously supplied uridine-C^{14}. As will be described in a later

paper there is in fact a by-pass around the main pyrimidine nucleotide pool for the entry of exogenous uridine-C^{14} into 50S RNA (McCarthy and Britten, 1962).

In calculating ϕ_{50} and ϕ_{40} the values of 1 and 2 per cent were used for their respective fractions of the total cellular RNA (Table II). If these values were incorrect, the curves for ϕ_{50} and ϕ_{40} would merely be shifted up or down along the ordinate. No change would be effected in the initial slopes of the curves or in the resulting argument that they are sequential precursors of ϕ_{32}. Nevertheless, it is possible to make an independent estimate of the amount of ribosomal RNA precursor in the following way.

From sucrose gradient analyses of RNA obtained by the phenol-SDS extraction method ratios of $\Sigma C^{14}/\Sigma OD_{260}$ were obtained, excluding 4S RNA. Similar ratios were derived from analyses of RNA isolated by the phenol-EDTA method, again excluding 4S RNA. The former ratios were essentially the specific activities of total cellular RNA, excluding 4S RNA, and the latter were the specific activities of ribosomal RNA. These specific activities were plotted against τ. Since the values for total RNA should fall on a curve described by $\mu(1-e^{-\tau})$, the constant μ was evaluated as described by McCarthy *et al.* (1962). After adjustment by this factor the points for total RNA should fit the curve $(1-e^{-\tau})$. Since the fit of the points to this theoretical line was good, as shown in Fig. 9, it is concluded that the system was essentially in steady-state and met the restrictive conditions imposed by Britten and McCarthy (1962).

There is clearly a large delay between ϕ_T and ϕ_R at early times. In fact the initial dependence of ϕ_R on τ^3 again indicates a lag in entry of label equivalent to two sequential precursors of ribosomal RNA. When the values of ϕ_R are subtracted from those of ϕ_T a curve ϕ_P results. This curve represents the over-all kinetics of labeling of those components of ϕ_T not included in ϕ_R, in other words the labeling of the 50S and 40S components. Since this curve, ϕ_P, levels off at $\phi = 0.04$, the amount of RNA represented by the 50S and 40S material is estimated at 4 per cent of ϕ_T, or about 3.4 per cent of the total cellular RNA in agreement with the estimate of 3 per cent in Table II. However, the maximum level reached by ϕ_P represents an upper limit on the amount of such precursors, and not necessarily the absolute amount. As will be discussed shortly there is evidence that ϕ_P represents a good deal of material that is not, at least directly, precursor of ribosomal RNA.

One further interesting point about the labeling of ribosomal RNA should be made. Reference to Fig. 7 shows that the 18S component is labeled at a slightly faster rate than 32S RNA. ϕ_{18} is not shown in Fig. 8 for the sake of simplicity but, when plotted in this way, the curve deviates slightly from ϕ_{32} and has an initial slope approximately equivalent to $\tau^{2.7}$. The meaning of this result is not clear, but at least it indicates that ribosomal RNA is not a kinetically homogeneous material. Perhaps labeled precursor first enters into 18S RNA which is then in equilibrium with the 32S component.

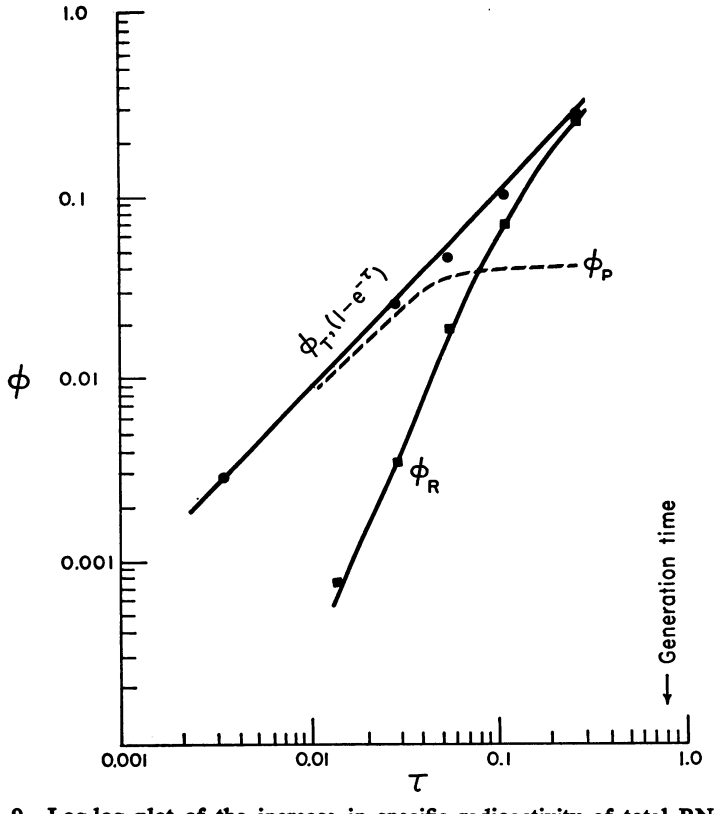

FIGURE 9 Log-log plot of the increase in specific radioactivity of total RNA (ϕ_T) and ribosomal RNA (ϕ_R) with time. The time, τ, is defined by $Q = Q_0 e^{\tau}$.

The analysis just performed supports the hypothesis that the 50S and 40S RNAs are sequential precursors of ribosomal RNA. On the other hand, a good argument can be made that the experimental data on labeling of these two components are not the most accurate, that both components might be heterogeneous, and that the observed kinetics are purely fortuitous. At its extreme, this argument would postulate that the delay in entry of label into ribosomal RNA is occasioned entirely by the lag in equilibrating a large nucleotide precursor pool, and that 50S and 40S RNA are not on the pathway to ribosomal RNA but have some other function. This explanation is probably incorrect in that it would seem to require a precursor pool of pyrimidine nucleotides larger than is estimated to exist in L cells (unpublished data). The more likely possibility that 50S and 40S RNA are synthesized and then broken down into more or less private ribosomal RNA precursor pools is difficult, if not impossible, to elucidate on kinetic grounds alone. There is, however, some information from experiments of a different type that help to answer the question.

For example, the results of Perry (1962) and of Scherrer *et al.* (1963) indicate that, in the presence of actinomycin D, entry of labeled exogenous precursor into ribosomal RNA is blocked. When actinomycin D is added after the cells have been labeled for 30 minutes or more, part of the incorporated label can then be "chased" into ribosomal RNA. These results indicate that actinomycin prevents the synthesis of a nuclear precursor of ribosomal RNA, but once the precursor has been synthesized, does not inhibit its entry into the ribosomes. The precursor material may break down en route, but at least not to the level of the nucleotide pools. Harris' recent results (1963) suggest quite the contrary, that actinomycin D blocks the entry of labeled nuclear RNA into the ribosomes and that this labeled material breaks down instead. It is possible, however, to reconcile these two apparently conflicting sets of results. 10 minute labeling periods were used by Harris while Perry and Scherrer *et al.* labeled their cells for 30 minutes or more. It is clear from the present work that only the 50S component would contain an appreciable amount of label after a 10 minute period. If it is assumed that 50S RNA contains two components, one that is labeled and then breaks down and another that is labeled, perhaps more slowly, and is a direct precursor of 40S RNA and ribosomal RNA, the data are explicable. With longer labeling periods the two components of the 50S fraction would be more fully labeled and breakdown in the fraction would be observed as well as some transfer of label into ribosomal RNA. In fact, it is known that some breakdown does occur in the rapidly labeled RNA of the cell nucleus (Harris and Watts, 1962; Harris *et al.*, 1963; Scherrer *et al.*, 1963; Harris, 1963) and our recent results (unpublished) indicate that this breakdown indeed occurs in the 50S component. The hypothesis that 50S and 40S nuclear RNA contain two sequential precursors of ribosomal RNA seems, therefore, to be strongly supported and is in general agreement with the conclusions of McCarthy *et al.* (1962) that there are two sequential steps in the synthesis of ribosomal RNA in *E. coli*. Some breakdown of these precursors in mammalian cells must occur to reduce their size of ribosomal RNA. Whether there is even more extensive breakdown followed by reassembly of the units is unknown.

The kinetics of labeling of the 4S fraction are difficult to explain. From Fig. 7 this material is labeled at a good deal faster rate than a metabolically stable fraction, represented by ribosomal RNA (Rake and Graham, 1962) and 4S RNA therefore undergoes some breakdown into its nucleotide precursor pools. Probably the 4S fraction contains more than one component, and it is in fact possible to approximate its kinetics on the assumption that it contains equal amounts of a metabolically stable component and another that is labeled at four times the rate. When ϕ_4 is plotted against τ the initial slope of the function is approximately $\tau^{1.7}$. Kinetically it is thus possible to postulate a precursor of ribosomal RNA in the 4S fraction. At any rate, there seems to be one precursor stage between the nucleotide pools and part of the 4S RNA, and a likely candidate for this precursor would

be the 50S nuclear component. One might speculate that the DNA-like RNA recently isolated by Hoyer *et al.* (1963) from mammalian cells is the fraction of nuclear RNA that undergoes breakdown, that it is part of the 50S nuclear component, and that its breakdown provides precursor for the 4S RNA. If, on further work, this proves to be the sequence, a reasonably unified concept of the synthesis of RNA in bacteria (McCarthy *et al.*, 1962; Midgely and McCarthy, 1962; Midgely, 1963) and mammalian cells would result.

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