SEDIMENTATION AND AUTORADIOGRAPHIC ANALYSES OF RAPIDLY LABELED RIBONUCLEIC ACIDS IN HUMAN AMNION CELLS

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ABSTRACT When human amnion cells were exposed to radioactive cytidine for 0.002 or 0.017 of one generation time, the alcohol-insoluble label in the RNA preparations from these cells was distributed between a rather homogeneous component of 4 to 8S and a fast mixture of 34S, 30S, 25S, 20S, and 15S. Evidence has been presented that these sedimenting components are RNA. More than 73 per cent of the label in the fast mixture from the cells labelled for 0.017 of one generation time was derived from the nuclei. The label in nucleotides after 0.017 of one generation time equaled 1.4 times that in RNA. Thus, the previous autoradiographic evidence for the nuclear origin of late cytoplasmic label is weak. The distribution of label among the various sedimenting components, as well as that between two pyrimidine nucleotide constitutents of 34S, 30S, 25S, and 15S components, changed when the length of exposure to radioactive cytidine was increased from 0.002 to 0.017 of one generation time. This result excluded the possibility that the population of the RNA labeled after 0.002 of one generation time was identical with that labeled later. This fact must be included in formulation of hypotheses for the function of rapidly labeled RNA's.

INTRODUCTION

The term rapidly labeled ribonucleic acids, as used in this paper, refers to ribonucleic acids (RNA's) which are found labeled after amnion cells in a late phase of active growth have been exposed to radioactive cytidine for 5 or 40 minutes at 37°C in a humidified chamber gassed with a mixture consisting of 5 per cent CO_2 and 95 per cent air. According to the autoradiographic results of Goldstein and Micou, when the cells were exposed to H^3-cytidine under such conditions, they were very slightly labeled after 2 minutes, only nuclear RNA was labeled after 5 minutes (1), and all the label in RNA was found in the nucleus after 60 minutes (2). The amount of labeled RNA decreased in the nucleus with an increase in the cytoplasm upon further incubation of the cells in a non-radioactive medium for 8 hours or longer

(2). The label in RNA was defined as the residual radioactivity of such cells fixed immediately with methyl alcohol and then digested with DNAase. Similar time and location sequence of the appearance of the labeled RNA has been observed with several other plant and animal cells, with various materials used as RNA precursor and with various techniques for fixing the cells and for differentiating RNA from DNA (3–7). In the *Neurospora* experiment of Zalokar (7), the label in cytoplasm was demonstrated to be in microsomes. However, no sedimentation analysis of the rapidly labeled RNA's has been made with any plant or animal cells. In this paper, the results of such an analysis in combination with autoradiography with amnion cells are reported.

Such a combined study was thought to be of interest on two accounts: (*a*) If all or most of the rapidly labeled RNA's are located in the nucleus, the study could yield information on the presence in the nucleus of rapidly labeled RNA with certain sedimentation constants without pre-separation of the nuclei from the cells. (*b*) With biochemical evidence already at hand that the ribonucleoprotein particles in microsomes are the primary sites for cytoplasmic protein synthesis in liver cells (8, 9) investigators have postulated, partly on the basis of the time and location sequence described above, that the rapidly labeled RNA serves as the messenger carrying genetic information from the nucleus to a cytoplasmic structure on which specific proteins are synthesized. Knowledge of the size and physical heterogeneity of the messenger is of obvious importance to the understanding of the mechanism by which the information passes from gene to protein. Moreover, should the messenger be found in the rapidly labeled RNA, as is widely contended, heterogeneity in the sedimentation constant of the RNA would raise a question as to which sedimentation fraction, or fractions, is the messenger.

The reported autoradiographic evidence for the translocation of labeled RNA from nucleus to cytoplasm is difficult to accept, however, for two reasons: (*a*) It does not exclude the possibility that the observed localization of the RNA in the nucleus results from preferential removal of radioactive RNA from cytoplasm over that from the nucleus by the fixation procedure and subsequent treatments. In fact, experimental data suggesting that this possibility is a probability can be found in the literature. Harris (10) reported that after exposure of connective tissue cells from rat heart for 20 minutes to H^3-adenosine, the label in RNA which appeared over the nucleus was about 60 per cent instead of the nearly 100 per cent found by all previous workers with other cells. In Harris's work, the cells after methyl alcohol fixation were extracted with trichloroacetic acid before autoradiography. (*b*) As pointed out by Harris in the same study, it is impossible to obtain positive evidence for translocation of the labeled RNA from nucleus to cytoplasm owing to the complication introduced by the presence of a large pool of RNA precursors. In the present study on the location of the rapidly labeled RNA in amnion cells, attention has been directed to these difficulties.

MATERIALS AND METHODS

A fresh monolayer of human amnion cells, strain A 185 21C, employed by Goldstein and Micou for their autoradiographic studies (2) and kindly supplied by the latter, was incubated in a growth medium (2) containing 14 μc H^3-cytidine (specific activity 1 curie/mmole, Schwarz Laboratories Inc., Mt. Vernon, New York) or 7 μc C-2-C^{14}-cytidine (specific activity 2.5 μc/mg, Schwarz Laboratories) per ml of growth medium at 37°C for 5 or 40 minutes, 0.002 or 0.017 generation time. After incubation with radioactive medium, the cells were washed three times with Tyrode's solution, detached from the glass with 0.1 per cent trypsin, and sedimented by centrifugation in the presence of 20 per cent horse serum. The resulting cells will be referred to as "fresh cells." The "fixed cells" used for RNA isolation were prepared by suspending the fresh cells in approximately 150 volumes of absolute methyl alcohol for 8 minutes and sedimenting them by centrifugation at 300 *g* for 5 minutes. RNA was extracted from both the fresh and the fixed cells by phenol immediately after cell preparations were made. The time interval between the termination of the incubation and the first phenol extraction was 10 minutes for fresh cells and 24 minutes for fixed cells.

Unless specified otherwise, all the manipulations involved in the extraction of RNA, and the studies on the properties of the RNA preparations were carried out at 0-3°C under sterile conditions. The basal medium for RNA was 5 gm/liter sodium citrate.

The Gierer-Schramm procedure of extracting RNA with phenol (11) was modified to improve the stability and recovery of RNA. This modification produces an RNA preparation from mouse brain infected with Semliki Forest virus that is superior to the preparations obtained by several other modifications, as judged by the viral infectivity and protein contamination (12). The first extraction was carried out by homogenizing the labeled cells in a Waring blendor at 60 volts with 7 ml of 5 gm/liter sodium citrate and 7 ml of 80 per cent redistilled phenol in the citrate solution for two 10-minute periods separated by a 1-minute intermission. The homogenate was then centrifuged to separate the aqueous phase from the phenol phase, and the latter was washed with one-third volume of the citrate solution. The washing, combined with the aqueous phase, was further extracted with 0.5 volume of phenol by vigorous agitation for 10 minutes. The extraction was repeated once with 0.25 volume of phenol. The resulting aqueous phase was extracted five times with 2 volumes of ether and then freed of residual ether by bubbling purified nitrogen gas through it. The preparation contained 93 to 98 per cent of the radioactivity in the initial suspension of cells. The radioactivity left in the phenol phase was primarily in DNA.

Sedimentation studies of radioactive nucleic acids in an RNA preparation were performed in a Spinco model E analytical ultracentrifuge using a swingbucket rotor, Spinco SW-39, at 39,460 RPM. For a study of zone sedimentation velocity, 0.5 ml of the RNA solution was layered over 4 ml of the sodium citrate solution, varying in sucrose concentration from 12.5 per cent to 30 per cent, and was centrifuged for 6 hours. For the investigation of trail-boundary sedimentation, 4.5 ml of the RNA solution with a continuous concentration gradient in sucrose varying from 0 per cent at the meniscus to 6 per cent at the bottom of the centrifuge tube was centrifuged for 2 hours. For a study of effective density in CsCl solution, 5 ml of a solution of the RNA in CsCl was centrifuged for 92 hours. In each sedimentation velocity study, mouse brain RNA's of 30S and 20S (13) were used as references in evaluating the sedimentation constants of the radioactive nucleic acids. In the density investigation, the brain RNA's and the thymus DNA prepared by Simmons's procedure (kindly supplied by Dr. E. Carusi) were used

as indicators for RNA and DNA, respectively. At the end of a centrifugation, fractions were obtained from a centrifuged tube by collecting drops from the tube bottom for the zone sedimentation study and by sampling from the meniscus with pipettes for the trail-boundary sedimentation or density study. The fractions were assayed for radioactivity and absorption at 258 mμ.

The sedimentation constant of component x, whose position after centrifugation did not coincide with that of any indicator, was the average of two values, s_{x_1} and s_{x_2}, calculated according to the following equations:

$$s_{x_1} = 30 \ln \frac{r_x}{r_0} \Big/ \ln \frac{r_{30}}{r_0}$$

$$s_{x_2} = 20 \ln \frac{r_x}{r_0} \Big/ \ln \frac{r_{20}}{r_0}$$

In these equations, r_0, r_x, r_{20}, and r_{30} are the effective distances from the axis of rotation after centrifugation of the meniscus, x, 20S, and 30S, respectively, if the sedimentation were carried out in water. To obtain an effective distance, the distance traveled in the density-gradient column was corrected for the viscosity and density factors affecting the sedimentation velocity. A curve relating the product of these two correction factors to the position of the column was constructed by determining the viscosities and densities of the fractions from the column after centrifugation and taking the partial specific volume of RNA as 0.578 (14).

The 1 M NaCl precipitation of a RNA preparation prior to sedimentation analysis was carried out as follows: The RNA solution with the addition of the brain RNA at a final concentration of 1 mg/ml was maintained with 1 M NaCl for 20 to 24 hours. The precipitate and supernatant fractions were obtained by centrifugation at 3,000 g for 45 minutes.

To determine the amount of label in 1 M NaCl-insoluble RNA, the NaCl precipitate, described above, was subjected to reprecipitation once with NaCl, and the radioactivity in the pellet from the second precipitation was recorded. To examine the extent to which the activity so determined might be due to co-precipitation of small molecules such as cytidine with the brain RNA, a parallel precipitation was made using H^3-cytidine at the same activity instead of a H^3-RNA preparation. The following results were obtained: (*a*) The pellet from a second precipitation contained 97 to 98 per cent of the initial brain RNA and, in the parallel precipitation, contained 0.4 per cent of the initial H^3-cytidine which can be completely accounted for by the liquid associated in the pellet. (*b*) After correction for the activity probably associated with the liquid of a pellet, the activity in the pellet from reprecipitation of the H^3-RNA preparation was not less than that from the first precipitation. It is, therefore, concluded that the co-precipitation of cytidine contributes little to the values determined for the radioactivity in 1 M NaCl-insoluble RNA.

The radioactivity of H^3 or C^{14} in the solution was determined by mixing 0.1 ml of the solution with 17 ml of scintillation liquid (3 ml of ethyl alcohol, 14 ml of toluene, 56 mg of 2,5-diphenyloxazole, and 1.4 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene) and counting the disintegration of H^3 or C^{14} in the mixture with a Tri-Carb liquid scintillation spectrometer (Packard Instrument Company, Chicago). The fractions from the zone sedimentation study and an effective density study were freed of sucrose and CsCl, respectively, before being mixed with the scintillation liquid by the following procedure:

0.1 ml of a fraction was mixed with 0.1 ml of a solution consisting of 2 mg/ml of brain RNA, 0.2 N NaCl, and 5 mg/ml sodium citrate, and the mixture was maintained for 10 minutes with 0.4 ml of an alcohol solution containing 5 per cent by volume of 1 M HCl. The nucleic acids were then sedimented by centrifugation at 5,800 *g* for 15 minutes. Such a procedure quantitatively sedimented the brain RNA used and yeast-soluble RNA (kindly supplied by Dr. J. Goldstein of The Rockefeller Institute) as well as all the fractions from the zone sedimentation study except those fractions from the top 0.7 ml in the centrifuge tube. The radioactivity in the pellet after such an alcohol precipitation of a RNA solution was designated as alcohol-insoluble label and, according to the evidence presented later, was in RNA. The amount of label in the supernatant was recorded as the label in the nucleotides. The activity in a pellet was determined with a digest of the pellet obtained by incubating with 0.3 N KOH for 18 hours at 37°C.

The absorption of a solution at 258 mμ was the difference between the optical densities at wave lengths of 258 mμ and 320 mμ, measured in 3 ml cells with a Beckman DU spectrophotometer. The recovery of the radioactivity and that of the absorption at 258 mμ were 98 per cent or higher in all the fractionations.

Paper electrophoresis of a ribonucleotide preparation was carried out according to the procedure of Crestfield and Allen (15). To obtain the ribonucleotide preparation, 1 M NaCl precipitate of a RNA preparation was reprecipitated in 67 per cent alcohol and was then hydrolyzed by incubation with 0.3 N KOH at 37°C for 18 hours. The potassium was removed as precipitate of $KClO_4$ before electrophoresis, according to the usual procedure. The distribution of tritium on the paper after electrophoresis was determined as follows: Spots containing nucleotides were identified under ultraviolet light, and strips were cut from the paper along the electrophoretic path. The strip containing nucleotides was then sectioned normal to the electrophoretic path. After numbering of these sections in order, each section, with or without nucleotides, was immersed in 17 ml of scintillation liquid overnight, and the liquid, together with the section, was counted in the liquid scintillation spectrometer. In order to determine the background values, the counts were also made with three additional sections from an adjacent strip containing no nucleotide.

Autoradiographs of the cells were prepared by the procedure used by Goldstein and Micou (2). The differentiation between the nucleolus, non-nucleolus nucleus, and cytoplasm was facilitated by staining the cells with methyl green–pyronin after the films were developed. The values reported for the distribution of silver grains among these three cellular regions were the averages for 90 cells selected from 12 to 15 fields under microscope; more cells were selected for a field with greater cell population and as many localities per field as possible were represented. The two determinations made by two investigators on a sample gave practically the same results.

RESULTS

1. *Sedimentation Analysis of Label in the RNA Preparations.* When human amnion cells were exposed to radioactive cytidine, the alcohol-insoluble label after 40 minutes, 0.017 of one generation time, was distributed approximately as 14 per cent in a rather homogeneous component, *c* (4 to 8S), and 86 per cent in the fast mixture (Fig. 1*a*). The mixture had two major peaks, *a* (30S) and *b* (20S), and three minor peaks, *d* (34S), *e* (25S), and *f* (15S). The label after 5 minutes, 0.002 generation time, was located with *c* and the fast mixture in roughly equal

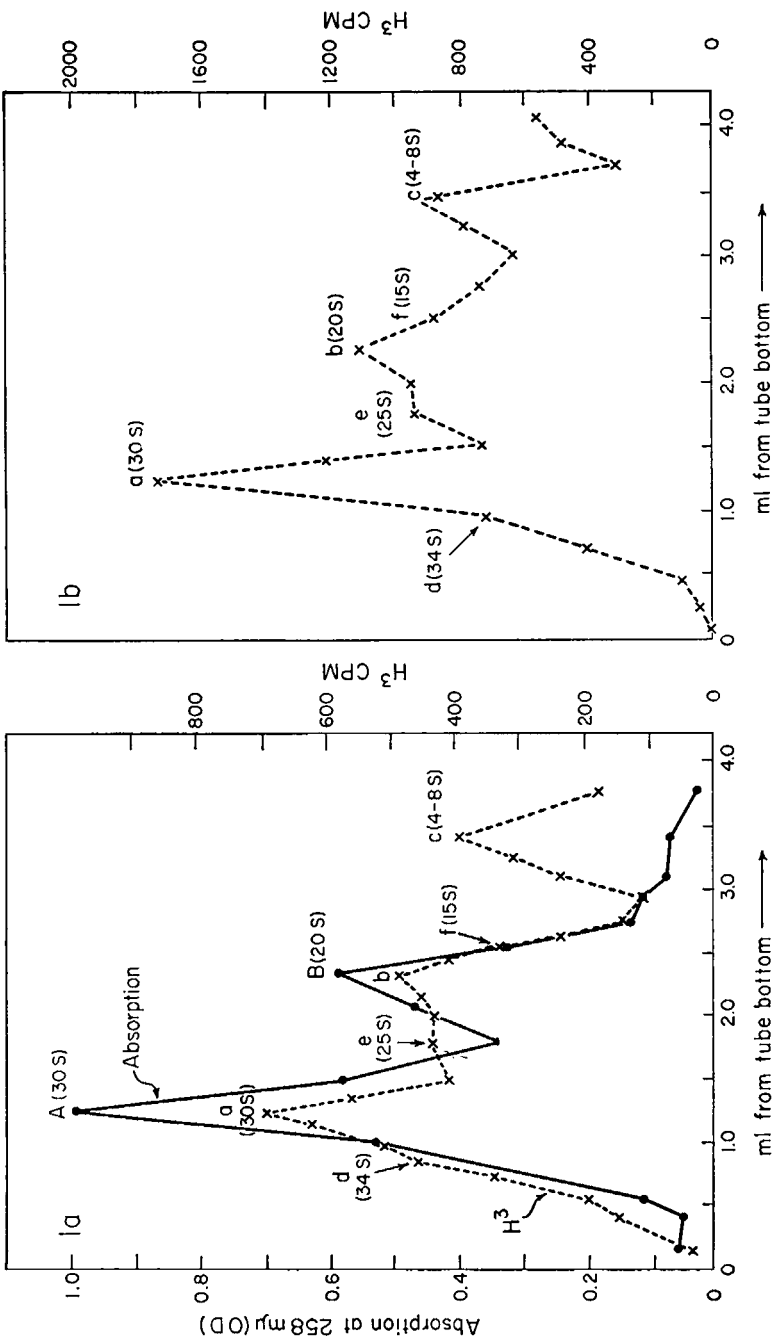

FIGURE 1 Zone sedimentation analysis of a RNA preparation from fresh amnion cells labeled for 40 minutes with H^3-cytidine. A (30S) and B (20S) are indicators. *a* (30S), *b* (20S), *c* (4 to 8S), *d* (34S), *e* (25S), and *f* (15S) denote labeled components. Fig. 1*a*, with indicators (dilution factor 0.4 ×); Fig. 1*b*, without indicator (dilution factor 1.0 ×).

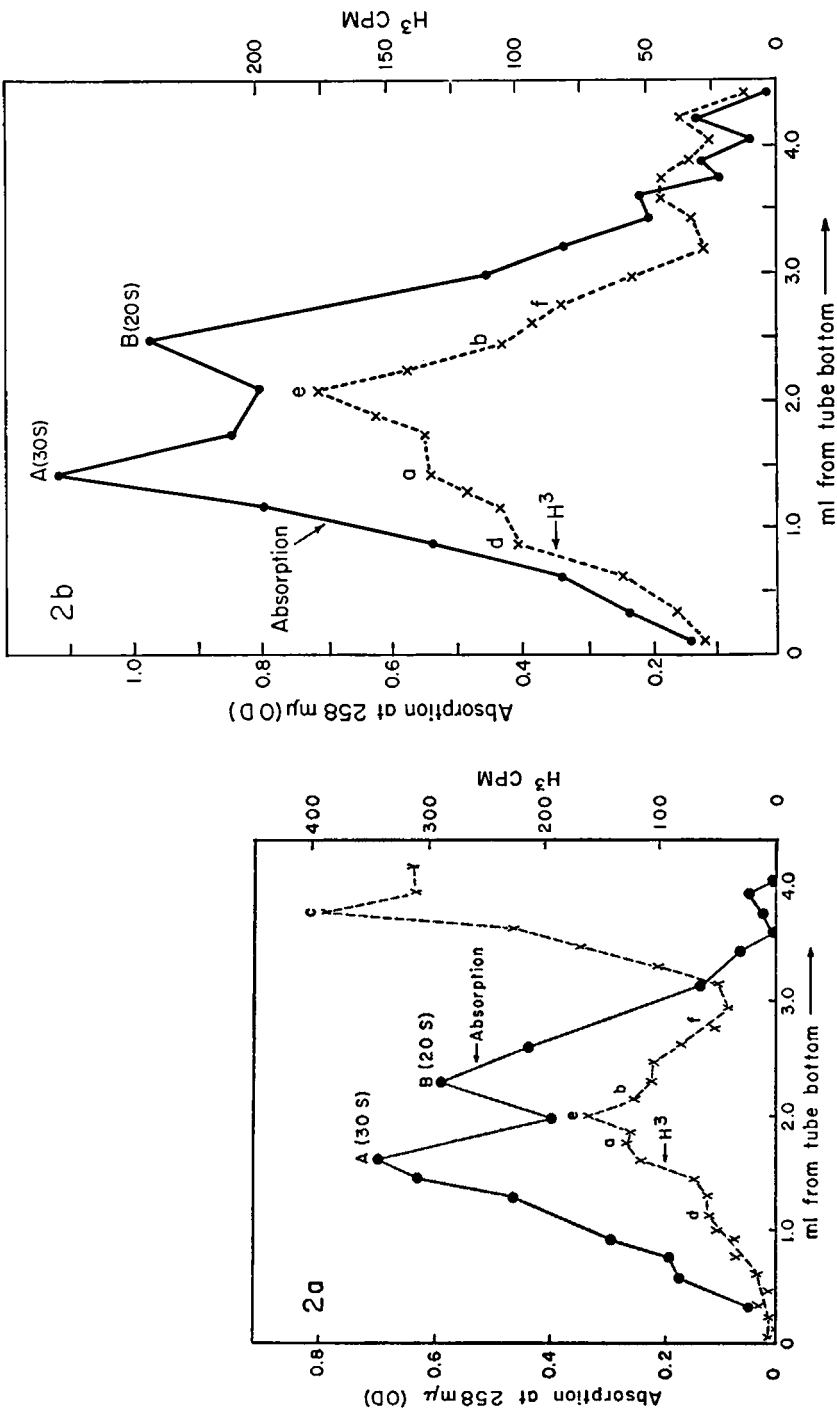

FIGURE 2 Zone sedimentation analysis of a RNA preparation from fresh amnion cells labeled for 5 minutes with H^3-cytidine. Fig. 2*a*, whole preparation (dilution factor 1.0 ×). Fig. 2*b*, precipitated with 1 M NaCl (dilution factor 1.2 ×).

amount (Fig. 2*a*). The mixture differed from that in Fig. 1*a* in two main respects: (*a*) the 20S, whose concentration was second only to 30S in Fig. 1*a*, became inapparent; (*b*) the 25S emerged as the most abundant component. The sedimentation patterns in these two figures are reproducible with the repeated RNA preparations. Peak *d* is better defined in Fig. 3 where peak *a* in the sedimentation pattern of the RNA preparation from cells labeled for 40 minutes was preferentially decreased through dialysis. The definition of both peaks *e* and *f* is improved in the sedimentation patterns of 1 M NaCl precipitates of the two RNA preparations (Figs. 2*b* and 4*a*), where the label preferentially left component *b*.

2. *Characterization of the Labeled Sedimenting Components.* It has become customary to assume, without obtaining adequate experimental evidence, that the label in sedimenting components obtained from a labeled RNA precursor is in RNA molecules. With the labeled sedimenting components in Figs. 1 and 2, three other possibilities were investigated:

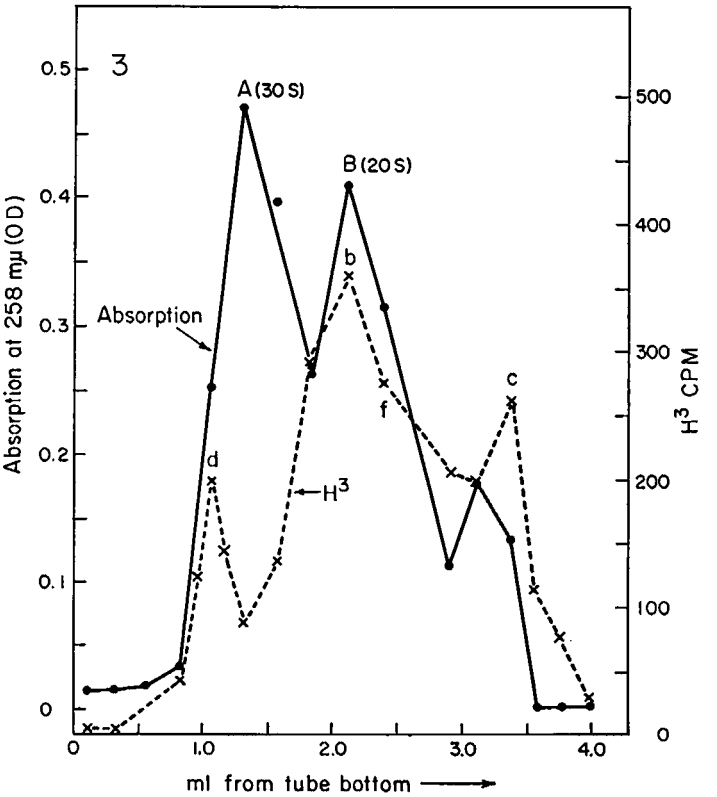

FIGURE 3 Zone sedimentation analysis of a RNA preparation from fresh amnion cells labeled for 40 minutes after dialysis of the preparation against 5 gm/liter sodium citrate for 22 hours at 2-3°C in the presence of indicator RNA's. The dilution factor for H^3-RNA was *ca.* 3.4 × that of Fig. 1*b*.

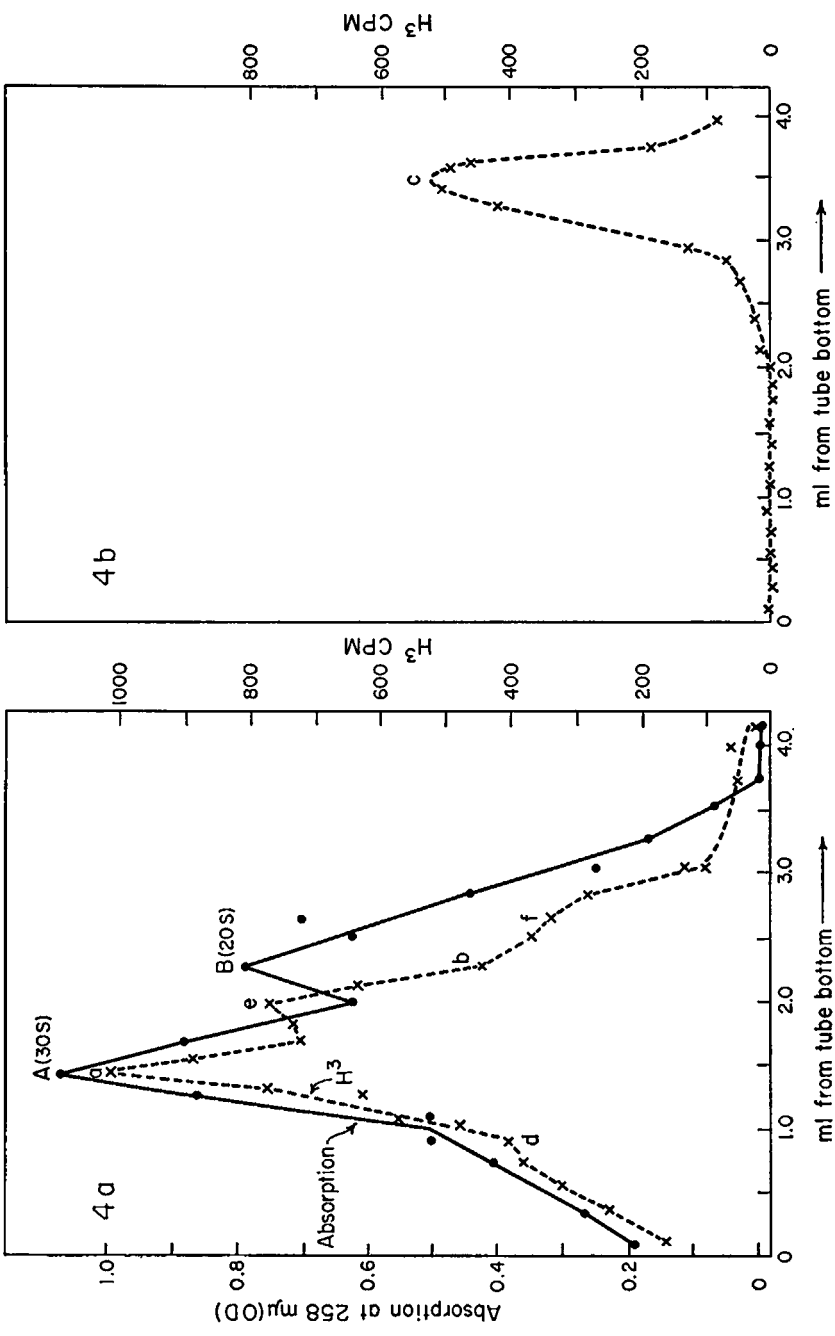

FIGURE 4 Zone sedimentation analysis of the two fractions of the RNA preparation in Fig. 1 after 1 M NaCl precipitation. The two fractions were centrifuged in the same rotor under the same conditions as Fig. 1. Fig. 4*a*, precipitate; Fig. 4*b*, supernatant. The dilution factors for Fig. 4*a* and Fig. 4*b* were respectively 1.2 × and 2.6 × that of Fig 1*b*.

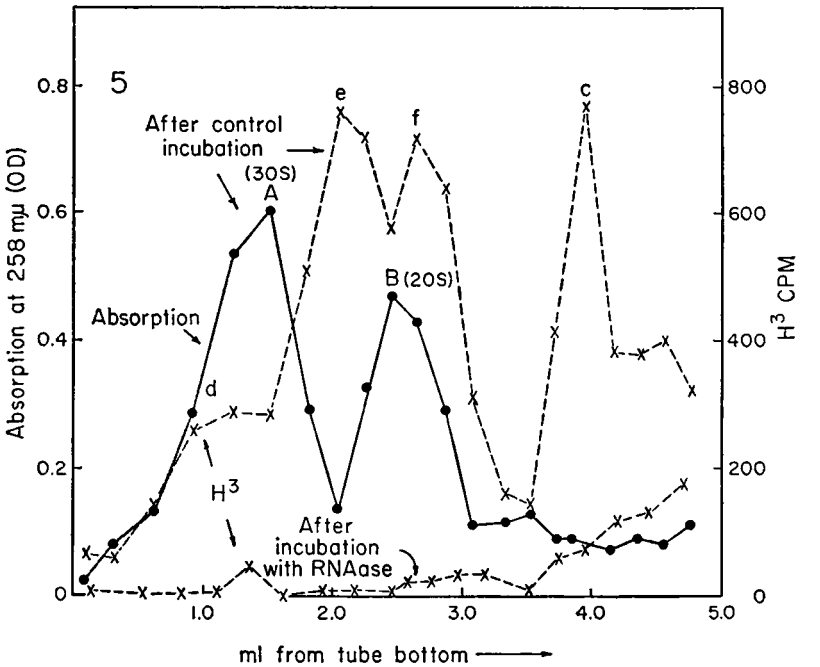

FIGURE 5 Zone sedimentation analysis of 40-minute–labeled nucleic acids after incubation with 0.05 M sodium phosphate, 3.75 mg/ml sodium citrate, and 1 mg/ml brain RNA, pH 7.0, for 50 minutes at 30°C with 50 γ/ml RNAase and without RNAase. The dilution factor of both samples was 2 × that of Fig. 1*b*. The absorption curve for the RNAase-treated sample is omitted. Alteration in the distribution of the label among the sedimentation components is also noted with the RNA preparation after the control incubation.

(*a*) The first of these was the possibility that some of the labeled sedimenting components were DNA because of contamination of the RNA preparation with DNA. When the RNA preparation from cells labeled for 40 minutes was incubated with ribonuclease, there was a loss of label from every sedimenting component (Fig. 5), a result indicating that all these components are RNA.

As previously described (13), the DNA contaminant in a RNA preparation obtained from mouse brain by a similar procedure is of high molecular weight and, in contrast to RNA's larger than 16S, is soluble in 1 M NaCl. Since this difference in solubility between the DNA and the RNA's is shared by preparations from all sources tested, including human cells from tissue cultures (16), it was used to determine the amount of H^3-DNA in these two fast mixtures. The two RNA preparations were subjected to 1 M NaCl precipitation, and the sedimentation studies were done on the resulting precipitate and supernatant. It can be seen from Fig. 4*b* that the supernatant of the RNA preparation from the cells labeled for 40 minutes contained no detectable amount of any component larger than 4 to 8S. A similar sedimentation pattern was obtained with the supernatant from the 5-minute–labeled

preparation. On the other hand, all the components in the fast mixtures except 20S appeared quantitatively in the 1 M NaCl precipitate (Figs. 2*b* and 4*a*). These results indicate that the two fast mixtures in Figs. 1 and 2 were only slightly contaminated with stable DNA of the molecular weights that have so far been isolated from various sources.

Further evidence that the components in the two NaCl precipitates were RNA was obtained by studying their effective densities in the CsCl density-gradient column. It can be seen from Fig. 6 that about 98 per cent of the H^3 was located with the indicator RNA's (at the tube bottom) and about 1 per cent was found either with thymus DNA or at some points in the column between the DNA and RNA bands. These data lead us to conclude that 34S, 30S, 25S, and 15S were RNA with very slight contamination of DNA and probably also RNA-DNA complex with a considerable percentage of DNA.

(*b*) The second possibility was that the label might simply be bound to the sedimenting components and not be *in* RNA molecules. A RNA preparation from cells labeled for 40 minutes was centrifuged in the presence and absence of added indicator RNA in the same centrifugation, the total RNA concentration of the former being 40 times that of the latter. As the sedimentation of H^3 gave a similar sedimentation pattern in both the presence (Fig. 1*a*) and the absence (Fig. 1*b*) of the indicator RNA's, none of these sedimenting components can be an artifact due to the presence of the indicator, for instance, adherence to the indicator.

As described under (*a*), the label in the mixture of 34S, 30S, 25S, and 15S remained with indicator RNA after NaCl precipitation and CsCl density-gradient centrifugation. In the first treatment the label was maintained with 1 M NaCl, pH 6.6, for 20 to 24 hours, while in the latter treatment it was exposed to 6.7 M CsCl, pH 7.1, at the beginning of centrifugation and at even higher concentrations of CsCl for most of the 92-hour centrifugation. The label was found with the indicator RNA under all the conditions we have tried, such as precipitation twice with 1 M NaCl, precipitation three times with 67 per cent alcohol, and maintenance at pH 1.6 for 10 minutes. Furthermore, as already described under Materials and Methods, cytidine was attached to indicator RNA to only a negligible extent after the second NaCl precipitation. None of these results favors the interpretation that the label was simply bound to the sedimenting components.

(*c*) The third possibility investigated was that the label in these sedimenting components might have arisen from a radioactive impurity in the sample of RNA precursor used instead of from the precursor itself. Paper electrophoresis was carried out with 0.3 N KOH hydrolysates of 1 M NaCl precipitates of both 5-minute–labeled and 40-minute–labeled RNA preparations. The results are presented in Table I. It can be seen that with both preparations the radioactivity was practically exclusively localized at the spots of cytidylic acid and uridylic acid.[1]

[1] Cytidine undergoes deamination in the cells to give uridine.

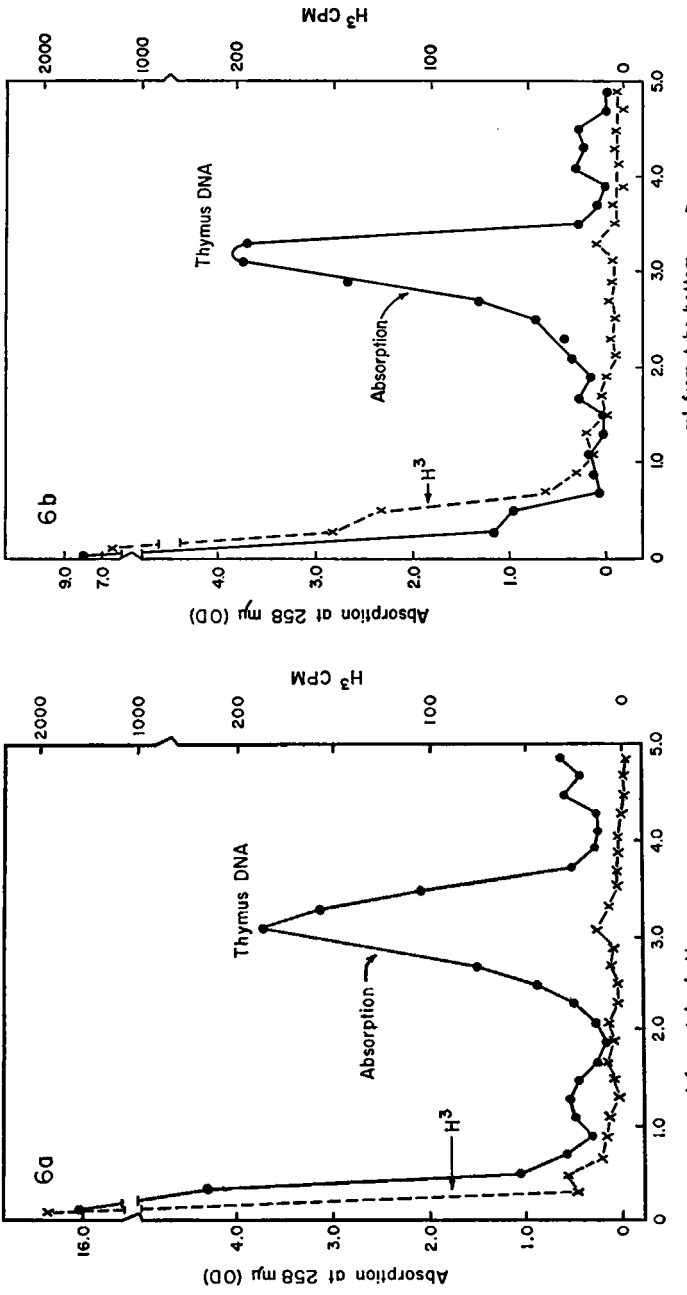

FIGURE 6 Analysis of effective density of 1 M NaCl-insoluble labeled components of a RNA preparation from fresh amnion cells near the sedimentation equilibrium of the RNA solution in CsCl. Thymus DNA added as a density indicator for DNA. Fig. 6*a*, 5-minute labeling with H^3-cytidine; Fig. 6*b*, 40-minute labeling.

Thus, the results of extensive characterization studies with the mixture of 34S, 30S, 25S, and 15S all point to the conclusion that these sedimenting components are RNA with the label in the RNA molecule.

With respect to (*b*) and (*c*), it may also be noted that the labeled precursor, when associated with such sedimenting components, coexisted with about a million-fold by weight of RNA under the experimental conditions, and that the label of such sedimenting components amounted to only a small fraction of the label in the

TABLE I

DISTRIBUTION OF H^3 ON PAPER AFTER ELECTROPHORESIS OF NUCLEOTIDE PREPARATIONS OF 1 M NACL-INSOLUBLE RNA'S FROM HUMAN AMNION CELLS LABELED WITH H^3-CYTIDINE*

Section No.	Ribonucleotide	H^3, counts/10 min.	
		5-Minute-labeled	40-Minute-labeled
1	None	310	326
2	Uridylic acid	601	644
3	Guanylic acid	302	332
4	None	323	308
5	None	287	294
6	Adenylic acid	303	339
7	Cytidylic acid	825	3366
8	None	295	312
	Average of background from adjacent paper strip	301	
H^3 in cytidylic/H^3 in uridylic‡		1.8	9.2

* Procedure in text.

‡ The value of H^3 in cytidylic (or uridylic) acid used in the calculation was the difference between the counts associated with cytidylic (or uridylic) acid spot and the background.

growth medium (1.3 per cent and 0.1 per cent in the case of 40-minute and 5-minute labeling, respectively).

3. *Composition and Location of Labeled RNA's.* Since the possibility that 4 to 8S could also contain small DNA or complexes of DNA or RNA with other material had not been excluded, the values estimated for the concentrations of 4 to 8S were taken as the maximum concentrations for the RNA with this sedimentation constant. By this calculation, at least 43 per cent and 86 per cent of the label in RNA were associated with the fast mixture in 5-minute–labeled and 40-minute–labeled preparations, respectively.

In order to determine the intracellular sites of these labeled nucleic acids by autoradiographic technique, the RNA preparation and autoradiographs of the cells must be made from cell populations having the same average distribution in the label. Autoradiographs of the 40-minute–labeled cells which were digested by a solution of DNAase in 0.003 M $MgSO_4$ immediately after fixation were first prepared by

strictly following the procedure of Goldstein and Micou (2). An amount of (90±13) per cent of the residual grain was found in the nuclei. Although the label was not exclusively located in the nucleus as previously reported (2), it was certainly predominantly found there. However, during the process of preparing such cells for RNA isolation, it was found that incubation of the fixed cells with 0.003 M $MgSO_4$ rendered many cells, and more than 90 per cent of the label, less sedimentable. This indication of leakage of material and label from the cells as a result of incubation certainly casts doubt on the validity of applying to living cells the autoradiographic data on the intracellular distribution of the label in DNAase-digested fixed cells. It also points to the difficulty of obtaining a cell preparation for RNA extraction with assured similarity in label distribution to the preparation for autoradiography.

For this reason, autoradiographs and RNA preparations of cells labeled for 40 minutes were obtained immediately after methyl alcohol fixation without further incubation. Grain count of such autoradiographs (Fig. 7) revealed the following distribution of the grain: (63±15) per cent in nucleoli, (28±9) per cent in the remainder of the nucleus, and (9±2) per cent in the cytoplasm. It should be noted, however, that the β-ray from H^3 disintegration in the cytoplasm encounters, on the average, a thinner layer of cell material before reaching the emulsion than a disintegration in the nucleus and, hence, has a higher probability of being recorded as a grain (17). Therefore, the percentage of the label in the cytoplasm was lower than 9 per cent, the percentage of the grain in the cytoplasm given here. The label in 1 M NaCl-insoluble RNA of such a RNA preparation was 33 per cent of the total label in the fixed cells. Thus, on the basis of the autoradiographic and chemical data, one can conclude that less than 27 per cent of the label in insoluble RNA of the fixed cells was present in the cytoplasm.

This intracellular distribution of insoluble-RNA label is believed to be similar to that in the fresh cells because of the following results: First, as seen in Fig. 8, the fixed cells gave a figure of 25 per cent for the insoluble-RNA label in terms of fresh cells, which is only slightly lower than the value of 28 per cent obtained with fresh cells. Second, in further similarity to that of fresh cells, the insoluble C^{14} was distributed primarily among 30S and a minor component whose *s* value did not differ greatly from 20S but was not accurately known because of the uncertainty involved in measuring the rather low radioactivity across the sedimentation boundary (Fig. 9). Thus, it would appear that methyl alcohol fixation neither greatly affected the amount nor irreversibly changed the physical state of the labeled insoluble RNA.

The percentage of the label of fast mixture in the cytoplasm was very probably considerably lower than 27 per cent for the following reasons: (*a*) Extensive biochemical data in the literature indicate that in the case of cytoplasm, 20S and 30S RNA's are much more slowly labeled than 4 to 8S RNA. Accordingly, one would expect only a small fraction of the cytoplasmic label to be in the fast mixture, bar-

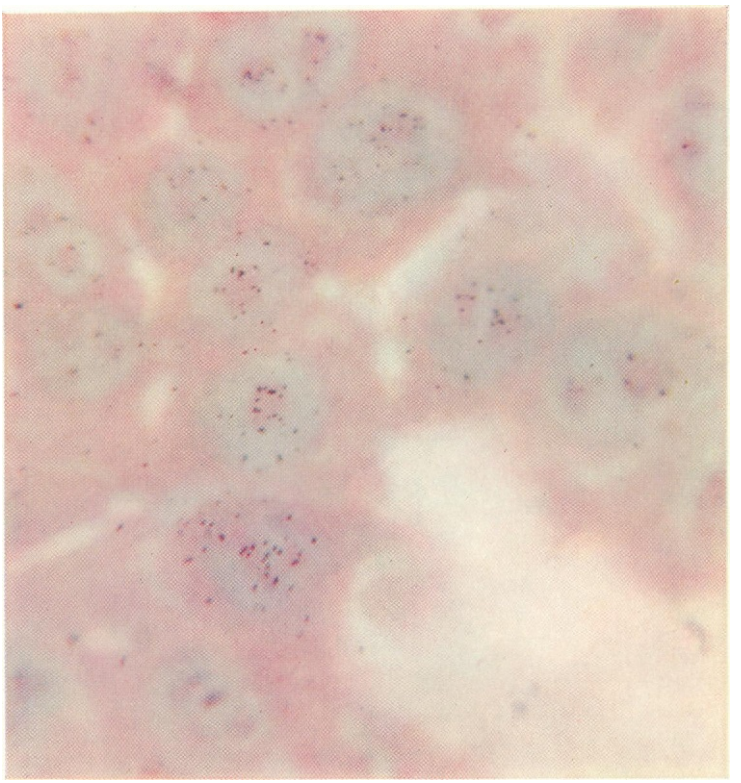

FIGURE 7 Autoradiograph of amnion cells labeled for 40 minutes with H^3-cytidine. Cells fixed with methyl alcohol.

ring the possibility that the label might be relocated during the isolation and fraction procedures involved. (*b*) If, as in the fresh cells, the label in insoluble RNA of the fixed cells was 80 per cent of that in the fast mixture (Fig. 8), then less than 22 per cent of the label in the fast mixture would be in the cytoplasm. Hence, it may well be that much more than 73 per cent of the label in the fast mixture was in the nucleus. The present chemical and autoradiographic data do not, however, permit us to decide the percentage of the nucleolus label in the fast mixture or the distribution of 4 to 8S label between nucleus and cytoplasm.

The amount of label in the nucleotides after the 40-minute labeling was 1.4 times that in the RNA. The presence of such a large pool of these precursors as a probable

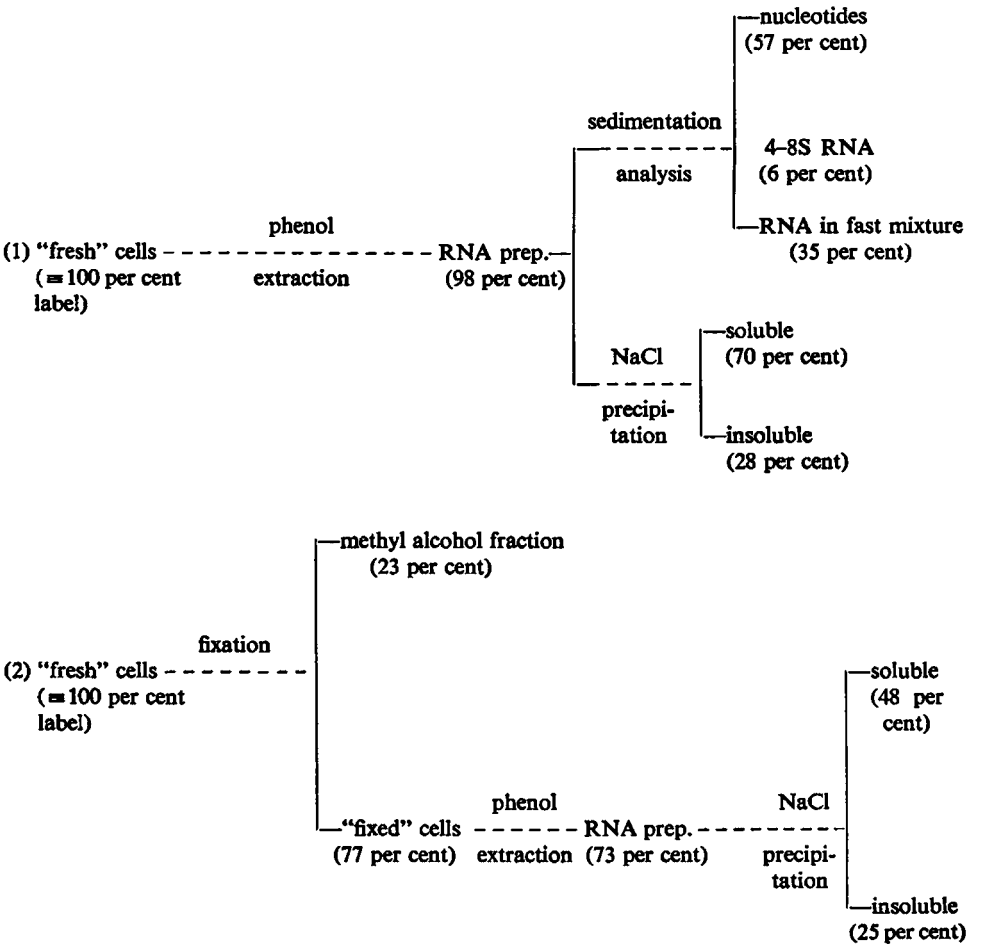

FIGURE 8 Diagram showing recovery of label in the fractions after various manipulative steps with amnion cells labeled for 40 minutes with tritiated cytidine. All percentages expressed in terms of fresh cells.

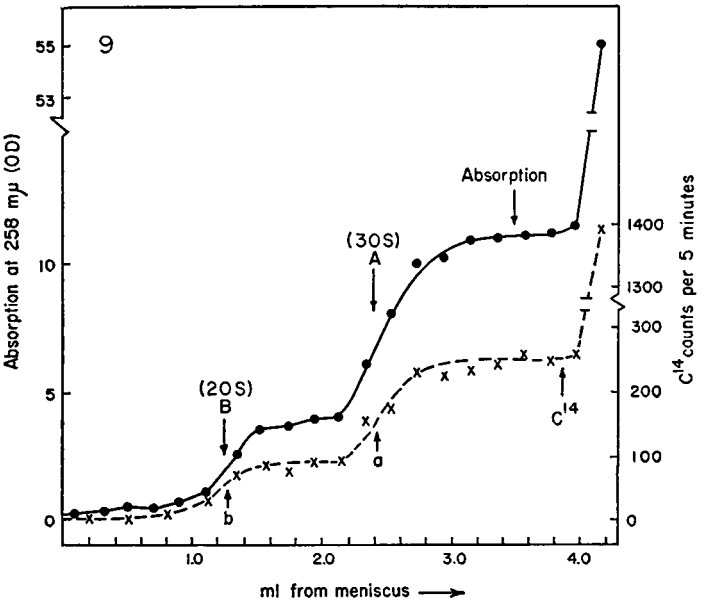

FIGURE 9 Trail-boundary sedimentation pattern of 1 M NaCl-insoluble C^{14}-RNA from fixed amnion cells labeled for 40 minutes with H^3-cytidine.

source for the late labeling of cytoplasmic RNA indicates the weakness of the previous autoradiographic evidence for the nuclear origin of cytoplasmic RNA in amnion cells (2).

The observation that H^3 is not associated with cytidine after perchloric acid hydrolysis of H^3-cytidine (L. Laufer, Schwarz Laboratories, personal communication) raises the possibility that some of the results described above may be an artifact due to instability of the H^3 label on the cytidine molecule. This possibility can be dismissed, however, on the basis of the following findings: (*a*) Parallel experiments with cells labeled with H^3-cytidine and those with C-2-C^{14}-cytidine have shown that the label distribution between the fractions from the H^3-labeled preparation agreed well with that between fractions from the C^{14}-labeled preparation after each step during the RNA preparation, *i.e.*, washing of cell layer, trypsinization, and three phenol extractions, as well as methyl alcohol fixation and washing of fixed cells. (*b*) As mentioned above, the sedimentation pattern of the label in insoluble RNA from C^{14}-fixed cells was similar to that from the H^3-labeled fresh cells.

DISCUSSION

RNA's of 30S and 20S have been isolated once before from the nuclei of calf thymus (18) and several times from microsomal particles or whole cells. However, extensive evidence has been accumulated that cytoplasmic RNA's of these sizes are

metabolically inert. The present finding constitutes the first instance in which RNA's of these sizes have been rapidly labeled. Furthermore, they are found in the nucleus. The fact that 30S and 20S RNA's have been found in both kinds of nuclei so far studied suggests their probable wide occurrence in the nuclei of various sources.

It is now widely believed that most of the cytoplasmic protein synthesis occurs in the 70 to 80S ribonucleoprotein particles and that the RNA preparation isolated from these particles consists of 30S and 20S particles. Although we see no reasons why the messenger cannot be of these sizes, nevertheless we think it possible that part of the rapidly labeled RNA's of 30S and 20S reported here were isolated from similar 70 to 80S particles from the nuclei of amnion cells. In the present study the RNA's labeled after 0.002 and 0.017 of one generation time differed in the distribution of label between two pyrimidine nucleotide constituents as well as in that among various sedimenting components. This finding excludes the possibility that the population of the RNA labeled after 0.002 of one generation time is identical with that labeled later, and hence is contrary to certain hypotheses for the function of rapidly labeled RNA's. It does not, however, rule out several others, including the interpretation that *all* molecules of the RNA labeled after 0.017 of one generation time are messengers.

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