An Analysis of the Rapidly Synthesized Ribonucleic Acid of the Normal Human Lymphocyte by Agarose–Polyacrylamide Gel Electrophoresis*

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ABSTRACT: Previous studies have demonstrated that the normal human lymphocyte *in vitro* rapidly incorporates labeled precursors into large molecular weight (30–100 S) non-rRNA which is heterodisperse in sucrose density gradient analysis. The development of mixed agarose–polyacrylamide gels for electrophoresis of RNA allowed a more detailed study of this material. Utilizing gel columns composed of 1.76% acrylamide, 0.2% bisacrylamide, and 0.25% agarose, a pore size is achieved which will readily admit and fractionate the large molecular weight RNAs synthesized by lymphocytes. Discrete peaks appear after about 1 hr of *in vitro* labeling, and by 6 hr, fractions of nominal molecular weights corresponding to 90, 75, 67, 50, and 40 S are consistently resolved. Cell fractionation and pulse-chase studies indicate that these molecules are turning over in the nucleus and do not bear a clear relationship to cytoplasmic heterodisperse RNA which requires much longer labeling periods to detect. Base ratio analysis of individual peaks demonstrates a high U content and (G + C) contents ranging from 36 to 50%. RNA extracted from different regions of the gel formed hybrids with homologous DNA to varying degrees. The greatest degree of hybrid formation occurred with RNA molecules larger than those in the discrete peaks. They were distributed in a heterodisperse fashion on the gel with molecular weights as high as 250 S. Stimulation of lymphocytes with phytohemagglutinin did not produce major qualitative changes in the monodisperse RNA peaks. The acrylamide gel technique has thus resolved several distinct molecular species of rapidly labeled nuclear RNA which were synthesized along with the more heterodisperse species and which could not be identified with other methods.

The nucleus of eucaryotic cells contains a heterogeneous class of large molecular weight RNA which undergoes rapid synthesis and degradation but whose function remains obscure. Its presence has been confirmed in many different cell types in several different laboratories (Scherrer *et al.*, 1966; Attardi *et al.*, 1966; Houssais and Attardi, 1966; Warner *et al.*, 1966; Penman *et al.*, 1966). Several properties have been described which characterize this material including heterodisperse (10–90 S) distribution on sucrose gradients, DNA-like (G + C) content, nucleoplasmic origin (Soeiro *et al.*, 1966; Scherrer *et al.*, 1966), and high efficiency of hybrid formation with homologous DNA (Perry *et al.*, 1964; Birnboim *et al.*, 1967). Despite these characteristics, several studies indicate that this material turns over in the nucleus, and that there is no clear precursor relationship to cytoplasmic polyribosome-bound mRNA (Attardi *et al.*, 1966; Soeiro *et al.*, 1968; Penman *et al.*, 1968). The experiments reported here were performed with small lymphocytes isolated from the blood of normal human subjects in short-term tissue culture. These small cells, composed of a compact nucleus and a thin rim of cytoplasm, are nondividing and do not synthesize DNA under standard conditions. Studies previously reported from this laboratory (Torelli *et al.*, 1968) demonstrated that the vast majority of the nucleic acids synthesized by these cells in short-term cultures were large molecular weight rapidly labeled heterogeneous RNA species with properties on sucrose gradient analysis which were indistinguishable from those described above for other cell types. Synthesis of rRNA was shown to be minimal in these cells, and therefore does not complicate studies of rapidly synthesized RNA.

The development by Peacock and Dingman (1968) of a series of mixed agarose–polyacrylamide gels with pore sizes large enough to admit and fractionate RNA molecules of molecular weight as high as 10^8 made available a method of fractionating populations of RNA molecules more completely than was possible with previously available techniques. In the hope of better characterizing lymphocyte heterodisperse nuclear RNA, we have applied a technique utilizing mixed acrylamide–agarose gel columns to the analysis of rapidly labeled RNA from normal human lymphocytes. The results of this study indicate that several apparently discrete, and heretofore undescribed, molecular species of low (G + C) content can be resolved by this technique and demonstrated to be turning over within the nucleus. These species of RNA are synthesized on a background of more heterodisperse RNA molecules which form hybrids with DNA with higher efficiencies than do the monodisperse molecular species.

Experimental Section

Materials

Nylon fiber (Leuko-Pak) was purchased from Fenwal Corp., Morton Grove, Ill., and thoroughly washed in distilled water to remove toxic materials. [5-^{3}H]Uridine (26 Ci/mmole), NCS solubilizer, and toluene–phosphor (Liquifluor) were obtained from Amersham-Searle Corp. [^{32}P]Phosphoric acid, carrier free, is a product of the New England Nuclear Corp. The water-jacketed disc gel apparatus was purchased from Büchler Instruments and thermally regulated by means of a refrigerated water bath with an external circulating pump.

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Acrylamide monomer and bis[1] were purchased from Kodak Corp. and recrystalized. TEMED, also a Kodak product, was redistilled. Agarose was "Seakem" distributed by Baush & Lomb. Cacodylic acid was purchased from Fisher Chemicals and recrystalized. The apparatus for horizontal slicing of the gel columns into 1.24-mm disks was made by Earl Sandbeck of Scientific Instruments, Baltimore, Md. Prepared thin-layer foils of MN-cellulose 300 were purchased from Brinkman Instrument Corp. Photoflow is a Kodak product. Phytohemagglutinin was Difco, PHA-P.

Methods

Preparation and Culture of Lymphocytes. Leukocyte-rich plasma was obtained by the sedimentation, at 37°, of heparinized fresh whole blood drawn from normal human subjects with 0.1 volume of 6% Dextran (mol wt 75,000) in normal saline. Polymorphonuclear leukocytes and monocytes were removed from the plasma by passage through a nylon fiber column according to a modification of the method of Greenwalt *et al.* (1962; Torelli *et al.*, 1968). The cells, 99% small lymphocytes, were deposited from the column effluent by centrifugation at 200*g* and suspended, at 37° in minimal essential medium (Eagle, 1959) supplemented with 20% autologous plasma, glutamine (0.3 g/l.), penicillin (10^5 units/l.), and streptomycin (100 mg/l.). In this medium, small lymphocytes were found viable by dye exclusion and capable of accumulating labeled precursor into RNA in linear fashion for periods of up to 48 hr (Torelli *et al.*, 1968). For [^{3}H]uridine labeling, 20 μCi/ml of the isotope was added. For ^{32}P labeling this isotope, in concentrations of 100 μCi/ml, was added to the cells in phosphate-free media and 20% autologous plasma dialyzed against phosphate-free media. For pulse-chase experiments, the cells were deposited from the medium containing isotope by centrifugation at 200*g* for 3 min and resuspended in identical fresh medium containing 20 μg/ml of cold uridine. Centrifugation in this manner does not affect the incorporation of labeled precursors into lymphocyte RNA (Cooper, 1968). The nucleic acid into which isotopic uridine is incorporated by human lymphocytes cultured in the manner described has been clearly identified in previous studies as RNA by sensitivity to alkaline hydrolysis (Torelli *et al.*, 1968), inhibition by actinomycin D (Cooper and Rubin, 1965; Kay, 1967; Neiman and Macdonnell, 1970), insensitivity to DNase (Rubin and Cooper, 1965), and failure to bind nitrocellulose filters (Neiman and Henry, 1969). Incorporation of [5-^{3}H]uridine into DNA in unstimulated lymphocytes could not be detected (Torelli *et al.*, 1968).

Extraction of RNA. Following incubation, cells were harvested by centrifugation at 200*g* and washed with cold normal saline and the RNA was extracted according to a previously described hot phenol–*m*-cresol–sodium dodecyl sulfate procedure for sucrose gradient analysis of lymphocyte RNA (Torelli *et al.*, 1968). The RNA pellet was washed once with 5 ml of cold 70% ethyl alcohol to remove residual salt and dissolved in distilled water (final concentration 5–10 mg of RNA/ml). In experiments where very small numbers of cells were extracted, unlabeled *Escherichia coli* RNA was added to the cells as carrier.

For experiments involving subcellular fractionation, the procedure was varied as follows: washed lymphocytes were suspended in cold hypotonic swelling solution (0.01 M Tris–0.08 M sucrose–0.0033 M $CaCl_2$, pH 7.4), 1 ml/10^8 cells, in a Dounce homogenizer and subjected to 10 strokes of a tight-fitting "B" pestle. The homogenate was made 0.1% with respect to Triton X-100 and 10 more strokes were applied. Following centrifugation at 200*g* for 5 min, the supernatant fraction was aspirated and extracted for cytoplasmic RNA in the usual way. The nuclear pellet was washed once in 0.15 M NaCl–0.01 M Tris–0.0033 M $CaCl_2$ (pH 7.4) and the nuclei lysed by suspension in hypertonic buffer (1.5 M NaCl–0.05 M $MgCl_2$–0.01 M Tris, pH 7.4). The gelatinous mass of DNA thus released was degraded for 2 min at room temperature by addition of 40 μg of electrophoretically purified deoxyribonuclease per 10^8 nuclei. The usual RNA extraction procedure was then carried out. By phase microscopy, this combination of cytoplasmic swelling and detergent treatment effectively strips the thin rim of cytoplasm of these small cells. This was confirmed by electron microscopic study (P. E. Neiman and P. H. Henry, unpublished observations) which also revealed the loss of the outer nuclear membrane. The retention in the nuclear fraction of 90–95% of the cellular DNA and the same percentage of radioactive RNA following a 15-min pulse label with [^{3}H]uridine indicates that there was less than 10% nuclear breakage by this technique.

Rous sarcoma virus 71S RNA labeled with [^{14}C]uridine, which was used as a marker, was obtained from transformed chick fibroblast cultures by a previously described method (Robinson *et al.*, 1965).

Agarose–Acrylamide Gel Electrophoresis of RNA. The method used in this study was adapted from that described by Kapodia *et al.* (1971). A 1.1% solution of agarose was prepared by boiling and then cooled to 50° in a temperature regulated water bath. For preparation of six gel columns, 5 ml of acrylamide monomer and bis in twice the final concentration required for the gel was mixed with 2.5 ml of cacodylate buffer (0.08 M cacodylic acid–0.04 M NaOH, pH 6.0) and deoxygenated by stirring for 5 min under a vacuum (243 mm). This solution was heated to 50° in the water bath and 2.25 ml of agarose solution was added and mixed. Finally, 0.25 ml of a fresh solution of 0.022% riboflavin and 0.6% ammonium persulfate along with TEMED, 15 μl for 2.0% acrylamide gels and 30 μl for 1.76% acrylamide gels, were added. The mixture was pipetted in 1.5-ml aliquots into acid washed, 7 × 10 mm glass columns precoated with 10% photoflow and fitted with rubber stoppers. In the case of 1.76% acrylamide, 0.2% bis gels the lower half of the columns was also precoated with 0.1% agarose to prevent the gels from falling out. A small layer of hot distilled water was layered above each gel column with a long needle and the gels were immersed in the filled lower buffer chamber of the electrophoresis apparatus precooled to 8°; 1-hr exposure to two opposing fluorescent light sources was allowed for polymerization. Following gelation, the rubber caps were removed and the upper buffer chamber was filled with 0.02 M cacodylate buffer (pH 6.0, identical with lower chamber buffer). RNA samples in 10 μl of water were mixed with an equal volume of 50% sucrose containing a bromophenol blue dye marker and layered on the upper surface of the gel column. The RNA concentrates at the sucrose gel interface and migrates in the gel toward the cathode under the influence of a current of 3 mA/gel.

Analysis by Gel Electrophoresis. Bulk species of RNA can be localized by staining for 15 min in 0.4% new methylene blue–0.4 M acetic acid–0.4 M sodium acetate followed by 18-hr destaining in cold running water. Examples of stained gels from electrophoresis of *E. coli* and lymphocyte marker RNAs are seen in Figure 1. As has been previously described (Pea-

[1] Abbreviations used are: bis, *N,N'*-methylenebisacrylamide; TEMED, *N,N,N',N'*-tetramethylethylenediamine; SSC, standard saline citrate buffer, 0.15 M NaCl–0.015 M sodium citrate.

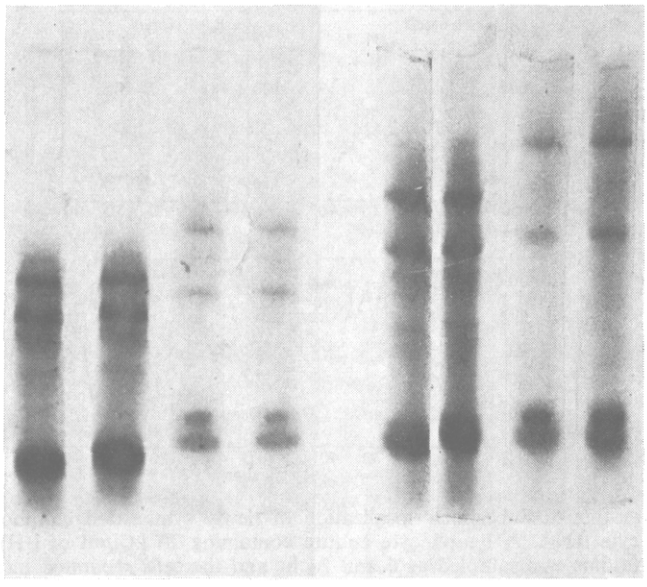

FIGURE 1: *E. coli* and human lymphocyte RNA on acrylamide gels. RNA (50 μg) from each of the cell types was applied to each of a pair of gel columns containing 1.76% acrylamide (first four gel columns) or 2.0% acrylamide (second four gel columns). The electrophoresis was carried out for 30 min at 3 mA/gel and the RNA bands were visualized by staining with new methylene blue. *E. coli* RNA is seen on the first and fourth pair of gels. The order of migration of the major bands seen on the gels from top to bottom was lymphocyte 28S RNA, *E. coli* 26S RNA, lymphocyte 18S RNA, *E. coli* 16S RNA, and 4S RNA.

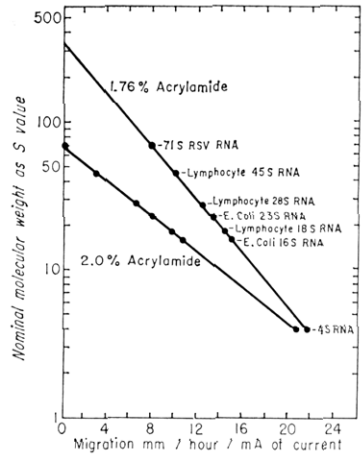

FIGURE 2: Determination of molecular weight on acrylamide gels. The migration of RNA bands from electrophoresis such as shown in Figure 1 is a linear function of the log of the molecular weight. *s* values are used here for convenience in comparison. The 45S marker is derived from experiments such as depicted in Figure 9 and the 71S RSV marker is illustrated in Figure 3.

cock and Dingman, 1968), the migration of an RNA band is directly related to the log of its molecular weight. The method of estimating molecular weight on the gels used in this study is illustrated in Figure 2. The localization of radioactive RNA species was obtained by slicing the gel, which had been chilled at 4° for 30 min, into 1.24-mm fractions. The slices were then incubated at room temperature with 0.5 ml of NCS solubilizer for 30 min and diluted to 5 ml with toluene–phosphor. Radioactivity was measured in a Packard TriCarb scintillation spectrometer. Variation in the volume of each slice can produce artifactual peaks of radioactivity. Uniformity of the slicing technique was determined by mixing radioactive RNA with the gel components and casting the gel column with uniformly distributed [^{3}H]RNA. The radioactivity of the slices from the column was assayed as a measure of slice volume and was found to vary by less than 10%.

Extraction of RNA from the Gel Slices. For purposes of performing RNA–DNA hybridization studies or molar base ratio analyses, the RNA was extracted from individual gel slices. The slices were finely minced with a sharp spatula, suspended in 2 ml of 0.1 M sodium acetate and 0.1 M EDTA (pH 5.1), and shaken with 1 ml of phenol at 60° for 3 min. The mixture was cooled to 4° and layers were separated by centrifugation at 880*g* for 5 min. The dispersed gel particle appeared as a white precipitate at the phenol aqueous interface. About 60–70% of the original radioactivity in the gel slices could be recovered by precipitation from the aqueous phase by the addition of 0.1 ml of 3 M NaCl and 2 volumes of cold ethyl alcohol. This mixture was allowed to stand at −20° for a minimum of 1 hr. Generally, 100 μg of an unlabeled carrier RNA was added prior to alcohol precipitation. The loss of radioactivity was largely accounted for by handling losses (reduction in volume of the aqueous phase) and equilibration with water dissolved in the phenol phase.

Base Composition. Molar base ratio analysis of [^{32}P]labeled RNA was performed by hydrolysis of the RNA pellet in 0.3 N potassium hydroxide for 18 hr at 37°. Following neutralization with perchloric acid, the constituent nucleotides were separated by thin cellulose-layer electrophoresis in pyridine–acetate buffer (pH 3.45) (Sebring and Saltzman, 1964) as previously described (Cooper, 1968). Average base composition studies on unfractionated RNA were performed after passage of the sample through Sephadex G-50 to remove contaminating labeled nucleotides.

RNA–DNA Hybridization Studies. Labeled RNA extracted from gel slices for hybridization measurements was incubated in 0.1 ml of 2X SSC, 0.05% sodium dodecyl sulfate at 67° with DNA extracted from human lymphocytes by the method of Marmur (1961). A characterization of the hybridization reactions of lymphocyte nucleic acids and the procedural details of this modification of the technique of Nygaard and Hall (1963) have been previously described (Torelli *et al.*, 1968; Neiman and Henry, 1969).

Results

Fractionation of Rapidly Labeled RNA. Figure 3 illustrates the migration on 1.76% acrylamide, 0.2% bis, and 0.25% agarose gels of radioactive RNA extracted from whole lymphocytes following a 6-hr pulse label. With this labeling time five fractions are consistantly resolved. The degree of reproducibility of this pattern is indicated by superimposing the distribution of radioactivity from two separately cast gel columns. Molecular weights corresponding to 40, 55, 67, and 75 S were assigned to the peaks and 90 S to the small shoulder on the 75S peak by reference to the labeled 71S RSV RNA marker and the 23S and 16S *E. coli* RNA markers which were stained on a column run in parallel with the radioactive RNA.

Labeling Kinetics. Figure 4 illustrates the development of these peaks after various periods of labeling. At 5 min, only minimal quantities of heterodisperse radioactivity could be detected. At 30 min, a small heterodisperse population of labeled molecules was detected ranging from 40 to 150 S with the suggestion of the appearance of 90S and 75S fractions.

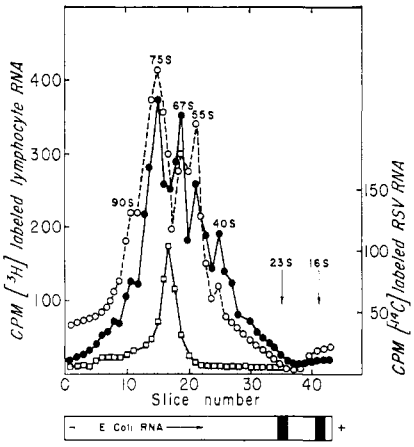

FIGURE 3: Acrylamide gel electrophoresis of lymphocyte rapidly labeled RNA following a 6-hr pulse label. A fresh lymphocyte culture was labeled with 10 μCi/ml of [^{3}H]uridine for 6 hr and RNA extracted from the cells and suspended in 50 μl of distilled water. Gel columns containing 1.76% acrylamide were cast in two separate batches. [^{3}H]Lymphocyte RNA (10 μl) (●—●) were mixed with 1 μl of ^{14}C-labeled 71S RSV RNA (□—□) and placed on a column from one gel batch and a second 10-μl aliquot of [^{3}H]-lymphocyte RNA (○—○) was layered on a gel column from the second batch. *E. coli* RNA (50 μg) was run on parallel gel columns. Electrophoresis was carried out of 3 mA/gel for 70 min. Radioactivity was located by slicing the gels and assaying the gel slices by the double-label-counting technique. The *E. coli* marker RNAs were identified by staining.

By 1 hr 90S, 75S, 67S, and 55S peaks were visible, and all five fractions were present at 3 and 6 hr. Figure 5 demonsrates the intracellular localization of the newly synthesized RNA. The additional handling of the nuclear pellet results in some degradation of its constituent radioactive RNA as evidenced by a smaller mean molecular weight than that observed with whole cell preparations and by the loss of definition of the peaks on gel electrophoresis. Nevertheless, it is apparent that for periods of up to 6 hr, the bulk of the labeled RNA species are confined to the nucleus. By 24 hr, however, some large molecular weight heterodisperse RNAs with a maximum concentration around 50 S in size are apparent in the cytoplasm.

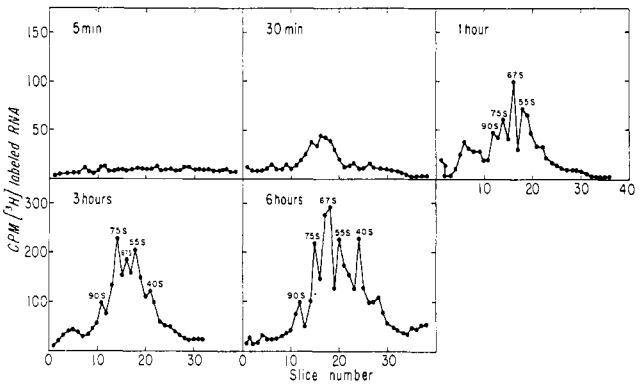

FIGURE 4: Acrylamide gel electrophoresis of lymphocyte RNA labeled with [^{3}H]uridine. A lymphocyte culture containing 20 μCi/ml of [^{3}H]uridine was sampled at various periods of time. The cells were extracted and their RNAs analyzed by electrophoresis on gels containing 1.76% acrylamide for 60–70 min at 3 mA/gel. Greater than 90% of the radioactivity applied to the gel is recovered. Molecular weights were assigned by staining paralled gel columns cast and run simultaneously with those used for radioactive RNA as in Figure 3.

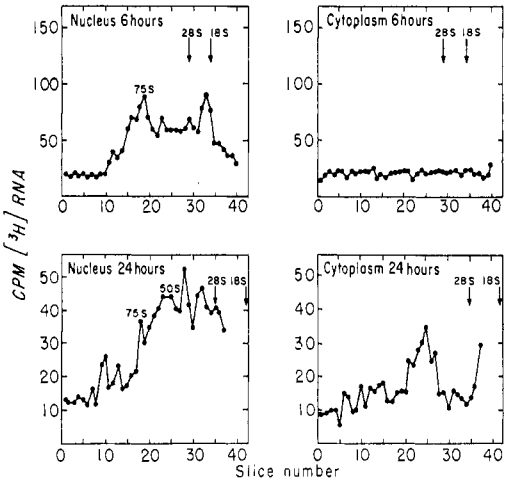

FIGURE 5: Subcellular localization of newly synthesized lymphocyte RNA. A lymphocyte culture containing 20 μCi/ml of [^{3}H]-uridine was sampled at 6 and 24 hr and the cells separated into nuclear and cytoplasmic fractions as in Methods. The radioactive RNA was analyzed on 1.76% acrylamide gels as in Figure 4.

The bulk of radioactive lymphocyte RNA will not migrate well on gels with pore sizes smaller than those of the 1.76% acrylamide preparation. This allows the analysis of very small amounts of labeled species with lower molecular weights. Figure 6 depicts the appearance of a group of slowly synthesized smaller molecular weight, monodisperse nuclear RNA species on 2.0% acrylamide, 0.2% bis, and 0.25% agarose gels. These are not apparent at 6 hr, but are easily detectable at 24 hr.

Pulse-Chase Experiments. An attempt was made to follow the posttranscription metabolism of labeled RNA species by replacing the culture medium containing isotope with fresh medium containing unlabeled uridine. The distribution of radioactive RNA in 1.76% acrylamide gels 3 and 6 hr after a 1-hr [^{3}H]uridine pulse is seen in Figure 7. Most of the radioactive peaks had largely decayed by the end of the chase period. Figure 6 also depicts the distribution on the gel of labeled RNA from whole cells, nucleus, and cytoplasm following a 6-hr pulse and 18-hr chase. RNA peaks synthesized during the first 6 hr of culture have been totally degraded by 24 hr. The large molecular weight RNA which slowly accumulates in the cytoplasm by 24 hr (Figure 5) does not appear, therefore, to have been derived from any easily identified nuclear RNAs which are present at 6 hr.

Hybridization and Base Ratio Measurements. RNA was

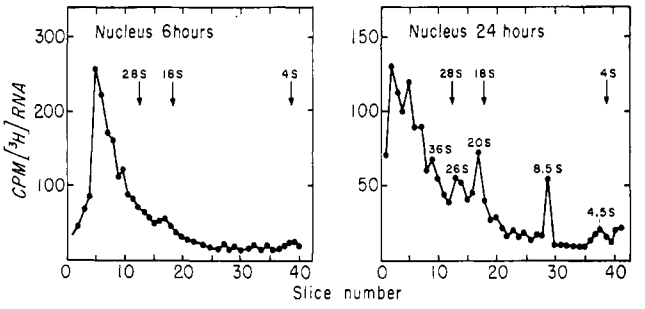

FIGURE 6: Smaller molecular weight slowly synthesized lymphocyte nuclear RNA. Lymphocyte nuclear RNA was obtained as in Figure 4. Electrophoresis was carried out with mixed gels containing 2.0% acrylamide at 3 mA/gel for 30 min.

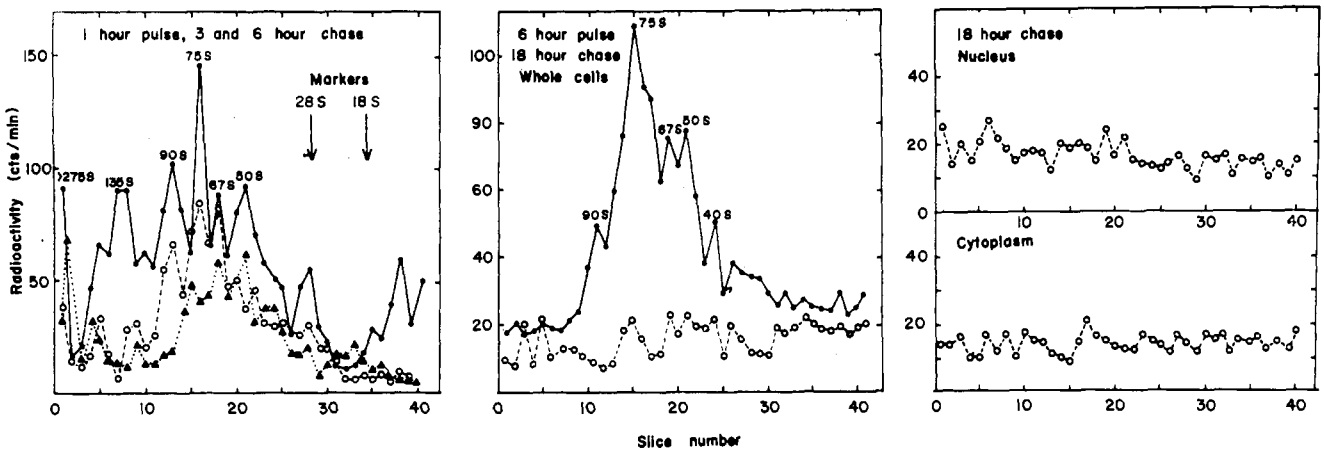

FIGURE 7: Pulse-chase experiments. Lymphocytes were incubated in medium containing 20 μCi/ml of [^{3}H]uridine. At the end of the pulse-labeling period, the cells were removed from the medium and resuspended in fresh medium as described in Methods. Left-hand panel: cells were pulse labeled for 1 hr (●—●), and an aliquot was extracted. Aliquots were extracted at 3 hr (○—○) and 6 hr (▲.....▲) during the chase period. The radioactive RNA was fractionated on gels containing 1.76% acrylamide as in Figure 3. Middle and right-hand panels: Cells were extracted after a 6-hr pulse label (●—●). After an 18-hr chase period, RNA from whole cells, nuclear and cytoplasmic fractions (○—○), was obtained and electrophoresced on gels containing 1.76% acrylamide.

extracted from individual gel slices for purposes of further analysis. Utilizing a ^{32}P label, both base ratio analysis and hybridization reactions can be performed with RNA extracted from a single gel column. Such an experiment is depicted in Figure 8. The pattern usually seen with isotopic uridine incorporation is also apparent after 6-hr ^{32}P labeling. Selective extraction of different species of RNA was not apparent in that the pattern of recovered radioactive RNA closely resembled that of a second gel column from which the gel slices were simply counted as usual. The RNA recovered from each slice was divided into two aliquots and hybridization reactions and base ratio analyses were carried out. The distribution of complementary RNA is depicted on a separate panel, and the hybridization efficiency of the RNA from each slice is plotted against the distribution of total extracted RNA. The analysis of the hybridization reaction demonstrates that the radioactivity of the monodisperse RNA peaks entered into hybrids with DNA at a lower efficiency than the 14.1% average for the entire RNA population. The most reactive species, with efficiencies of up to 30%, are distributed in heterodisperse fashion with molecular weights as high as 250 S. It should be noted that these hybrid reactions were performed at an RNA:DNA ratio of nearly 0.1 with respect to the entire RNA sample added to the gel column. This ratio has previously been shown to yield maximum hybridization efficiencies with unfractionated lymphocyte radioactive RNA under the conditions employed in this study (Torelli, 1968; Neiman and Henry, 1969).

The base compositions of the various peaks from this same experiment are displayed in Table I. The average base composition of the whole population of ^{32}P-labeled RNAs is DNA-like with respect to its low (G + C) content (43.6% for the total population and as low as 36% for some fractions). The relatively high U content seen here in some fractions has also been noted in other laboratories as a feature of heterodisperse nuclear RNA in other cell types (Scherrer *et al.*, 1966; Soeiro *et al.*, 1966; Cooper, 1968).

Effect of Phytohemagglutinin Stimulation. Under the influence of mitogens such as phytohemagglutinin, the human small lymphocyte undergoes major structural and functional change resembling immune reactivity (Nowel, 1960). Within hours after the exposure to the mitogen RNA and protein synthesis are markedly enhanced. By 24 hr DNA synthesis has begun followed by enlargement of the cell and mitosis. Organization of a prominent nucleolus has been correlated with a massive increase in ribosomal RNA synthesis underlying the bulk of the increase in RNA accompanying this phytohemmagglutinin-induced transformation (Torelli *et al.*, 1968). We examined the rapidly labeled RNA of transforming lymphocytes in an attempt to detect qualitative changes which might be correlated with structural and functional changes in the cell. Parallel cultures with and without added phytohemagglutinin were pulse labeled with [^{14}C]- and [^{3}H]uridine, respectively, following 24-hr culture. Their radioactive RNAs were extracted and subjected to coelectrophoresis on the

TABLE I: Base Composition of ^{32}P-Labeled RNA Fractions from Acrylamide Gels.[a]

Fraction Base (S)	U (%)	A (%)	G (%)	C (%)
300–140	36.1	26.8	20.1	17.0
115	31.5	22.5	29.9	16.1
90	26.4	23.6	24.9	25.1
75	29.2	26.7	26.0	18.1
67	25.1	34.1	19.7	21.1
50	30.8	27.1	22.6	19.5
40	30.9	27.7	27.0	14.4
Average	29.0	27.4	24.0	19.6

[a] Base ratio measurements of lymphocyte RNA labeled for 6 hr with ^{32}P as in Figure 8. 300–140 S fraction represents pooled RNA extracted from slices in that region of the gel column. The rest of the fractions are derived from single slices. Average: these figures are derived from an aliquot of [^{32}P]RNA taken from the sample just prior to gel electrophoresis which was run through Sephadex G-50 and then analyzed as in the section on Methods.

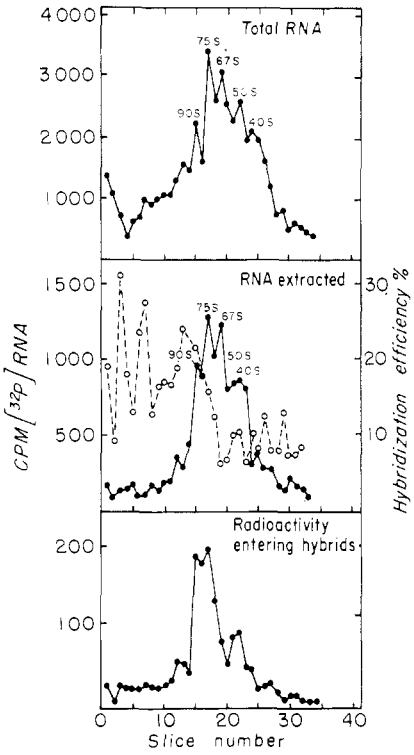

FIGURE 8: RNA–DNA hybridization of ^{32}P-labeled lymphocyte RNA. Lymphocytes were incubated for 6 hr in phosphate-free medium containing 100 μCi/ml of [^{32}P]phosphoric acid, and then extracted for RNA. The upper panel depicts the distribution of total labeled RNA on gels containing 1.76% acrylamide following electrophoresis for 70 min at 3 mA/gel. The middle panel indicates the distribution of the labeled RNA extracted from each of the slices of a second simultaneously processed gel column (●—●) and the efficiency of hybridization (total cpm hybridized/total extracted cpm) × 100 of the radioactivity of each slice with lymphocyte DNA (○—○). The lower panel indicates the radioactivity from each slice which entered the hybrids with DNA. ^{32}P-labeled RNA (7 μg) was applied to each gel. The RNA from each slice was coprecipitated with 100 μg of *E. coli* RNA, and the hybridization reactions were carried out in 0.1 ml of 2X SSC, 0.05% sodium dodecyl sulfate at 67° for 16 hr with 70 μg of lymphocyte DNA.

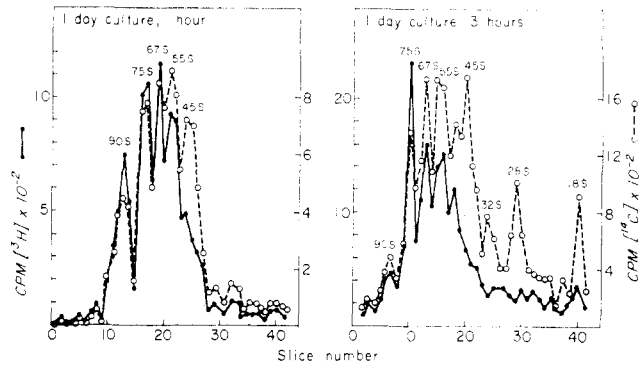

FIGURE 9: Effect of phytohemagglutinin stimulation. Parallel 15-ml cultures of lymphocytes, one with 2.5 μl/ml of phytohemagglutinin added, were incubated at 37° for 24 hr. [^{14}C]Uridine (1 μCi/ml) was then added to the stimulated culture and 10 μCi/ml of [^{3}H]uridine was added to the control culture. At 1 and 3 hr following labeling, 7-ml aliquots were removed from each culture, the cells washed, combined, and coextracted, and the RNAs subjected to coelectrophoresis. Double label counting technique was used to identify ^{14}C-labeled RNA from stimulated culture (○—○) and ^{3}H-labeled RNA from control culture (●—●) in the gel slices.

same gel column. Figure 9 illustrates the results of this experiment. RNA peaks at 90, 75, 67, 55 S were identified in both unstimulated and transforming cultures following labeling periods of 1 and 3 hr. Although an increased rate of incorporation of radioactive uridine into all RNA species was noted in transformed cultures, no gross qualitative differences in the newly identified RNA species were apparent. Rapidly labeled RNA species appearing only in the transformed cell cultures were noted at 45 S following 1-hr labeling and at 45, 32, 28, and 18 S at 3 hr. By 6-hr labeling mature r- and tRNA species dominate the pattern of radioactive RNA as shown in Figure 10.

Discussion

Previously reported studies have suggested that improved fractionation of the nucleoplasmic RNA species may be possible by polyacrylamide gel electrophoresis (Dingman and Peacock, 1968; Willems *et al.*, 1969). We have chosen to apply this technique to examine RNA synthesis in populations of isolated human lymphocytes in short-term tissue culture. We did so because this system offers a good opportunity for the study of the large, rapidly synthesized nuclear RNAs in normal, nondividing diploid cells. The experiments described above indicate that the synthesis of at least five apparently discrete, and heretofore undescribed, peaks of RNA can be detected during labeling periods of 6 hr. Molecular weights corresponding to 90, 75, 67, 55, and 40 S were tentatively assigned to the various peaks on the assumption of a linear relationship between electrophoretic migration and the log of the molecular weight. These RNA species band sufficiently close together that the imposition of 1-mm slicing resolves only the apex of the peaks above the background of overlapping bases and heterodisperse radioactivity. Thus the peaks are often found in single slices. The contention that these are discrete peaks and not simply random departures from a heterodisperse base line rests on three observations: (1) the calibration of variability of slice volume as described in Methods, (2) the repetition of the pattern in many experiments, and (3) a comparison of band widths of known monodisperse RNA species in double-label experiments. In addition to the example given in Figure 3 the degree of reproducibility of the RNA peaks is indicated in eight other electrophoretic patterns included in this report. The peak widths for 71S Rous sarcoma virus RNA (Figure 3), 45S and 32S pre-rRNA, and 28S and 18S rRNA (Figures 9 and 10) were noted and their apical portions appear compatible in width with those of the newly described RNA peaks. Nevertheless, the pattern is quite complex. The 90S peak tends to become buried with labeling periods of greater than 3 hr, while the 40S peak requires labeling periods of greater than 3 hr for clear definition. Further, the gel patterns indicated a large residual pool of rapidly labeled heterodisperse RNA species with molecular weights corresponding to as much as 250 S. The distribution on the gel of these molecules undoubtedly overlapped that of the monodisperse species and may account in part for the variability seen in degree of separation and relative amounts of the peaks in different RNA preparations. Variability induced by minor differences in the metabolic state of different lymphocyte cultures may also play a role.

The newly synthesized RNA species seem to be confined to the nucleus, and there appears to be a much slower accumulation of large molecular weight heterodisperse RNAs in the cytoplasm although some degradation of the RNA accompa-

nied cell fractionation procedure in these studies. The pulse-chase experiments indicate that the monodisperse RNAs are largely turned over within the nucleus and do not appear to be related to the slowly accumulating cytoplasmic species. This conclusion regarding the apparent independent turnover of nuclear and cytoplasmic RNA matches that recently reported for the nuclear and cytoplasmic heterodisperse RNA species of the HeLa cell (Penman *et al.*, 1968) and the avian erythroblast (Scherrer *et al.*, 1966; Attardi *et al.*, 1966). The complex pattern of the RNA distribution on the gels and the sluggishness of the cold uridine chase technique precludes any comment on possible precursor–product relationships between higher and lower molecular weight peaks other than the absence of any clear-cut evidence for such a relationship in these experiments. Quantitative turnover data are difficult to obtain for the same reasons although crude estimates based on the above data suggest decay half-times varying from 40 min to 8 hr for the monodisperse peaks. The absence of incorporation of methyl groups into lymphocyte nuclear RNA (with the exception of the very small amount of ribosomal precursor RNA detected only with [*methyl*-^{3}H]methionine) has been previously reported (Torelli *et al.*, 1968). This appears to be a general property of the large molecular weight nucleoplasmic RNAs (Brown and Attardi, 1965).

The analysis of RNA separated from the gel slices was performed in an attempt to characterize more fully the RNA fractions obtained by polyacrylamide gel electrophoresis. The two analytic techniques applied to RNA extracted from gel slices in this study confirmed the presence of different populations of RNA molecules in the various regions of the gel column. Base composition measurements indicated that all ^{32}P-labeled species had low (G + C) contents in comparison to rRNA although considerable variation of the composition of the individual peaks was observed. Hybridization efficiency was also variable in different regions of the gel. More specifically, radioactivity from the region of the gel column occupied by the monodisperse peaks entered hybrids with lower efficiencies than the average for the entire population. The highest efficiency of hybridization was detected in a region of the gel occupied by heterodisperse RNAs of even larger molecular weights than those estimated for the peaks. If the molecular weights of the heterodisperse species overlapped those of the RNA peaks, they might be responsible for a substantial part of the hybridization reaction detected in the 90–40S region of the gel column. This seems likely, since the analysis of RNA from lymphocytes labeled for 30 min or less demonstrated a substantial pool of rapidly labeled heterodisperse RNA in this region of the gel. Thus, the actual monodisperse RNA species most probably forms hybrids with homologous DNA under these conditions, at even lower efficiencies than those indicated in the experiment. This might reflect saturation of their homologous DNA at lower RNA:DNA ratios than those required for polydisperse species or failure to enter hybrids under the reaction conditions because of transcription from DNA of unique base sequence as suggested by Britten and Kohne (1968).

The experiments with stimulated lymphocytes were performed in an attempt to obtain some clue as to the functional significance of the rapidly synthesized nuclear RNAs by correlation of qualitative change by gel electrophoretic analysis with the events of lymphocyte transformation. No new nonribosomal species could easily be identified. The monodisperse RNAs of unstimulated culture were noted in transforming cells along with an increased rate of incorporation of isotopic uridine. The relative contributions of increased RNA synthesis and increased labeled precursor production to this quantitative change were not studied. These experiments served to confirm the observation that rRNA production is dramatically enhanced in stimulated lymphocytes.

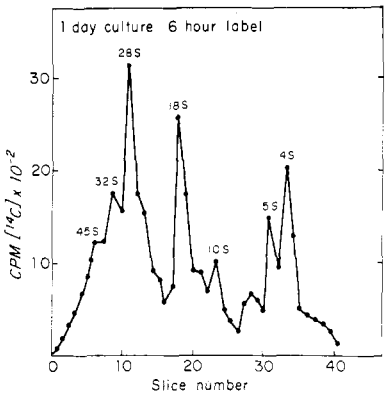

FIGURE 10: Gel electrophoresis of RNA from phytohemagglutinin-stimulated lymphocytes. Cells from a 24-hr lymphocyte culture stimulated with phytohemagglutinin were labeled for 6 hr with 1 μCi/ml of [^{14}C]uridine.

We conclude from these experiments that the rapidly labeled non-rRNA of the normal human lymphocyte which appears heterodisperse on sucrose gradient can be separated into two nuclear classes by polyacrylamide–agarose gel electrophoresis. The first is a group of monodisperse species partially resolved into five peaks varying from 40 to 90 S in size which are turning over in the nucleus. Their presence was also detected in lymphocytes transforming under the influence of phytohemagglutinin although no obvious qualitative changes in these new species were noted by gel electrophoresis. The second class of RNA is composed of a population of molecules with a heterogeneous distribution of molecular weights of up to 250 S in size. They formed hybrids with homologous DNA with an efficiency approaching the 30% reported for the reassociation of denatured DNA from mammalian sources under similar conditions (Britten and Kohne, 1968), and are thus probably derived from a broad spectrum of the genome. It remains to be seen whether or not the monodisperse nuclear RNAs described here in lymphocytes are specific for this cell type or are a general phenomenon in mammalian cells.

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