

Defective Control of Ribosomal RNA Processing in Stimulated Leukemic Lymphocytes

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ABSTRACT The kinetics of ribosomal RNA transcription and processing were assessed in chronic lymphocytic leukemia (CLL) lymphocytes during the initial phases of their delayed response to phytohemagglutinin. When compared to cultures of normal lymphocytes, CLL cultures developed normal augmentations in 45S rRNA precursor transcription and cleavage after a 1 hr incubation with PHA. However, failure to conserve 18S RNA subunits persisted in the CLL cultures. Subsequently the PHA-induced progressive rise in 45S RNA transcription became aborted and the over-all rate of RNA synthesis lagged far behind the levels attained by normal cultures incubated with PHA for 48 hr. CLL cultures responding to PHA in a delayed fashion exhibited efficient conservation of 18S RNA at 168 hr. In normal cultures, PHA-induced conservation of 18S RNA appeared to be independent of any effect on 45S ribosomal RNA precursor transcription. Therefore, the sluggish growth response of CLL lymphocytes was associated with a defect in one of the important mechanisms regulating assembly of new ribosomes.

INTRODUCTION

Suspensions of lymphocytes isolated from the blood of normal individuals respond to phytohemagglutinin (PHA)¹ in a predictable fashion (1). With PHA in the medium for 36–48 hr, 60–80% of the surviving lymphocytes transform into blast cells characterized by their large size, copious basophilic cytoplasm, finely dispersed nuclear chromatin network, and prominent nucleoli. Metabolically, this phenomenon is associated with a rapid increase in the rates of RNA and protein synthe-

sis over the first 48 hr (2). DNA synthesis begins at 36 hr and approaches a maximum at 72 hr when the cultures contain many mitotic figures (2). Circulating lymphocytes from patients with chronic lymphocytic leukemia (CLL) are functionally abnormal in that they show a delayed and quantitatively diminished response to PHA (2, 3). Thus, CLL lymphocytes require 120–168 hr to develop into blast cells active in RNA synthesis and DNA replication.

Analysis of morphology and of cell cycle kinetics has failed to identify any differences between the proliferating blasts derived from normal individuals and the late appearing blasts from CLL patients.² This implied that the delay occurs during transformation of the resting CLL lymphocytes (G_0) into blast cells (G_1) and that once CLL lymphocytes enter into the G_1 phase of the cell cycle, they proliferate normally.

Communications from our laboratory (4, 5) as well as the data reported by Cooper (6, 7) have demonstrated that transformation of resting lymphocytes into blast cells involves an increased efficiency of new ribosome production. Resting lymphocytes failed to show a net accumulation of new ribosomes due, in part, to a low level of 45S ribosomal RNA (rRNA) precursor transcription. However, these cells placed even greater restrictions on new ribosome production by limiting the processing of nascent 45S rRNA precursor molecules into mature 28S and 18S subunits destined for assembly into cytoplasmic ribosomes.³ Thus, in approximately 50%

² Johnson, L. I., J. LoBue, and A. D. Rubin. The lymphocytes of chronic lymphocytic leukemia: their proliferation and cell cycle kinetics. Submitted for publication.

³ As reviewed in reference 6, mammalian ribosomal RNA is transcribed in the nucleolus as a single precursor molecule characterized by its sedimentation rate of 45S. Each 45S precursor molecule undergoes cleavage into one 32S and one 18S subunit. The 18S subunit proceeds directly into the cytoplasm while the 32S subunit undergoes further intranuclear cleavage into a 28S moiety before joining the 18S subunit in the cytoplasm. Ultimately, one 28S and one 18S subunit comprise the RNA component of a mature cytoplasmic ribosome.

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¹ *Abbreviations used in this paper:* CLL, chronic lymphocytic leukemia; PHA, phytohemagglutinin; SDS, sodium dodecyl sulfate.

of 45S molecules transcribed by resting cells, the portion of the 45S molecule containing the precursor to an 18S subunit was rapidly degraded. After stimulation by PHA, an increase in the over-all rate of RNA synthesis, detected within 1–2 hr, rose to a maximal level at 48 hr. Simultaneously, these PHA-stimulated cells efficiently conserved 18S RNA subunits for the first 2–6 hr, after which wastage resumed. Pronounced wastage of 18S RNA reappeared in 48-hr cultures at the time when 45S rRNA precursor transcription was maximal and blast cells were plentiful in the culture. Therefore, the efficiency of ribosome assembly seemed to play a role in the initiation of cell enlargement. The present study explored ribosomal RNA transcription and processing in CLL lymphocytes during the first 48 hr of the delayed response to PHA. The data demonstrated an initial stimulation of 45S rRNA precursor transcription and processing in CLL lymphocytes but no conservation of 18S RNA. A subsequent rise in 45S RNA transcription became aborted as CLL lymphocytes failed to enlarge at 48 hr. Efficient conservation of 18S RNA took place at 168 hr in PHA-treated CLL cultures during the phase of delayed growth.

METHODS

Selection of lymphocyte donors. Healthy individuals whose ages ranged from 20 to 50 yr were accepted as lymphocyte donors. CLL patients fit our criteria for "high count" disease (3). These patients exhibited peripheral lymphocyte counts of greater than 50,000/mm³, hepatosplenomegaly, generalized lymphadenopathy, and diffuse bone marrow lymphocytosis. None of these patients received corticosteroids or cytotoxic agents within 1 month of the study. Patients meeting these criteria showed least variation in results. Isolated lymphocytes from all CLL patients employed in the study showed a delayed growth response to PHA (5–7 days) as previously described (2).

Isolation of lymphocytes. Blood was obtained from each donor by phlebotomy under sterile precautions. Gravity sedimentation provided a leukocyte-rich supernatant fraction which was then passed through a column of nylon fiber (5). This technique yielded suspensions of morphologically identifiable lymphocytes in 95–98% purity.

Lymphocyte cultures. Lymphocyte suspensions were adjusted to a concentration of 50×10^6 cells in 25 ml of Eagles No. 2 medium (adapted for spinner cultures). The medium contained added glutamine (2mmoles/liter), normal human type AB serum (15%), together with penicillin (100 U/ml) and streptomycin (100 U/ml). Difco PHA-p (19 µg/ml) was introduced and the cultures were incubated for specified times at 37°C in tightly stoppered bottles. Radioactive precursors, tritiated uridine (³HU, Schwarz 20 µCi/ml, 20 Ci/mmmole), or methionine-methyl-tritium (³HMe, Schwarz, 20 µCi/ml, 4 Ci/mmmole) were introduced at specified intervals before harvest. Before the introduction of ³HMe the cells were resuspended in methionine-free Eagles medium containing sodium formate (20 mmmoles/liter) and 10% calf serum. No cell counts or viability assessments were performed after initiating cultivation because of the extreme degree of agglutination

caused by PHA treatment. Nucleic acid extraction provided the only practical means of cell quantitation.

RNA extraction and quantitation. The cultures were harvested over a frozen slurry of Earles balanced salt solution. The extraction procedure involved lysis of all the cells in each culture in 0.5% sodium dodecyl sulfate (SDS) followed by a 5 min agitation with hot phenol according to a previously described technique (5). Precipitates consisting of the RNA extracted from whole cultures were dissolved in 0.2 ml of acetate buffer (0.01 mole/liter, pH 5.1) containing MgCl₂ (0.001 moles/liter), and NaCl (0.05 mole/liter). The final solution was layered on 5 ml of 5–20% sucrose gradient containing sodium acetate (0.01 mole/liter, pH 5.1) and NaCl (0.05 mole/liter). The gradient was rotated for 180 min at 37,000 rpm in an SW65 rotor of a Spinco model L-2 ultracentrifuge. Fractions were collected on filter paper discs on which the RNA was precipitated and washed with 5% trichloroacetic acid. Dehydrated discs were placed in a toluene cocktail for liquid scintillation counting. Optical density measurements of unlabeled rat liver RNA served as sedimentation rate markers at 28S and 18S.

The technique of phenol-SDS extraction of RNA so employed (5) isolated $85 \pm 5\%$ of the bulk RNA of resting lymphocytes as assessed by comparing the optical density of acid-precipitable (2% perchloric acid) phenol-SDS-extracted RNA with the optical density (at 260 mµ) of total acid-precipitable lymphocyte RNA (2). Identical yields were obtained by comparing radioactivity of phenol-SDS-extracted RNA with total acid-precipitable radioactive RNA after incubating the cells for 1, 2, 5, and 6 hr with either ³HU or ³HMe. The yield of radioactive RNA extracted by the phenol-SDS technique did not change over a 168 hr incubation with PHA. Furthermore, the 60°C phenol-SDS extract exhibited the same specific activity (counts per minute/optical density) as the small amount of unextracted acid-precipitable RNA remaining in the aqueous-phenol interface. Except for the occasional experiment in which a large amount of RNA tended to aggregate at the bottom of the centrifuge tube, $87 \pm 5\%$ of the acid-precipitable radioactivity layered on a sucrose gradient was recovered after sedimentation. Findings with CLL lymphocytes were identical. Therefore, the technique employed was considered reliable as a quantitative assessment of lymphocyte RNA synthesis in each culture.

Optical density measurements of total acid-precipitable RNA and DNA extracted from lymphocyte cultures set up in parallel showed no significant change over a 5 hr incubation with PHA. The technique employed for quantitation of acid-precipitable RNA and DNA differed from our previously published technique (2, 3) in that the fixation step was omitted. This alteration eliminated the PHA-induced loss of macromolecular RNA reported in a previous publication (8). After 48 hr incubation with PHA the optical densities fell to 50–70% and after 168 hr to 30–50% of resting levels. Findings with CLL lymphocytes were identical.

Phenol-SDS extracts isolated from both normal and CLL cultures, in the absence of rat liver carrier RNA, repeatedly displayed identical optical density patterns after sucrose density sedimentation. The areas under the peaks, relative to one another, were unchanged by prolonged incubation of the cultures with PHA. This confirmed previously published results (8) which demonstrated that bulk lymphocyte RNA sediments almost entirely in three distinct peaks at 28S and 18S, presumably derived from cyto-

plasmic ribosomes with a 4S peak consisting of transfer RNA along with some products of partial degradation.

Therefore, for at least the first 5 hr in culture with PHA, the bulk of lymphocyte RNA was assumed to be constant and subsequently all quantitative data for nascent RNA were expressed in terms of radioactivity alone.

Calculations. Radioactive RNA was quantitated by planimetric measurements of the area under well-defined peaks of radioactive methylated RNA (Me RNA) sedimenting at 45S, 32S-28S, and 18S (5). From available data regarding the molecular weights and degree of methylation of 28S and 18S rRNA subunits, an ideal ratio (r) for methylated 18S RNA (Me RNA_{18S}) divided by methylated 32S-28S RNA (Me RNA_{32S-28S}) was calculated as 0.67 assuming 18S RNA and 32S-28S RNA represented equimolar products derived from the cleavage of one 45S precursor molecule (6, 7). Ratios of less than 0.67 indicated 18S RNA wastage. Such wastage could be calculated and appropriate corrections made for estimating a true value for total 45S RNA precursor transcription. Thus, for estimating total 45S RNA transcription, Me RNA_{total}, the area under the observed peak (Me RNA_{45S}) was added to the area under the 32S-28S peak (Me RNA_{32S-28S}) plus an 18S peak (Me RNA_{18S}) corrected for wastage. With no wastage:

$$r = \frac{\text{Me RNA}_{18S}}{\text{Me RNA}_{32S-28S}} = 0.67$$

then

$$\text{Me RNA}_{\text{total}} = \text{Me RNA}_{45S} + \text{Me RNA}_{32S-28S} + 0.67 \text{ Me RNA}_{32S-28S}.$$

To express the augmentation in Me RNA_{total} induced by PHA treatment, the following ratio was employed:

$$R = \frac{\text{Me RNA}_{\text{total,PHA}}}{\text{Me RNA}_{\text{total,resting}}}$$

where Me RNA_{total,resting} refers to total 45S rRNA precursor transcription in resting cultures and Me RNA_{total,PHA} refers to total 45S rRNA precursor transcription in any culture treated with PHA.

All observations and calculations were based on cumulative data derived from at least six CLL patients and at least six age-matched controls. The sedimentation profiles presented in the figures depicted the results of one typical experiment.

RESULTS

48-hr cultures. During the height of the growth response to PHA, over-all RNA synthesis in 48-hr cultures of normal lymphocytes consisted, for the most part, of ribosomal species reflected in the predominating peak of 45S RNA labeled during a 15 min pulse of ³HU (Fig. 1a). During a 4 hr pulse (Fig. 1b), well-defined peaks of radioactive RNA appeared at 28S and 18S as the longer labeling interval allowed for complete processing of 45S rRNA precursor into mature species of 28S and 18S rRNA. In both of these patterns, most of the label was contained within 45S, 28S, and 18S peaks. In CLL cultures incubated with PHA for 48 hr, RNA labeled during a 15 min pulse failed to sediment in discrete

peaks (Fig. 1c). This phenomenon presented itself more clearly after a 4 hr pulse (Fig. 1d), where radioactive peaks of 45S, 28S, and 18S RNA were situated above a relatively large base of heterodisperse elements. A markedly diminished rate of ³HU incorporation is apparent in the PHA-treated CLL cultures.

Therefore, at 48 hr, cultures of PHA-treated normal lymphocytes could be distinguished from similarly treated CLL cultures by a relatively rapid rate of ³HU incorporation into RNA and a preponderance of rRNA accumulation over the accumulation of heterodisperse elements.

In these experiments the radioactive peak at the top of the gradient sedimented at 4S and represented, for the most part, transfer RNA. This fraction was not investigated as a part of the present study.

Initial phase of the PHA response. Radioactive RNA was accumulated by resting normal lymphocytes during a 2 hr pulse of ³HU sedimented in ill-defined peaks at 45S and 28S-32S (Fig. 2, left). These peaks were partially masked by a relatively large amount of heterodisperse RNA. As the heterodisperse moieties tend to turn over more rapidly than ribosomal moieties (5), it can be appreciated that in resting lymphocytes, incorporation of ³HU into rRNA was relatively slow. 1 hr after exposing resting lymphocytes to PHA, the amount of radioactive RNA accumulated during a 2 hr pulse doubled, but the peaks of radioactive rRNA sedimenting at 45S and 28S-32S became more prominent suggesting a selective stimulation in the synthesis of rRNA. A parallel experiment performed on CLL lymphocytes (Fig. 2, right) yielded similar results. The increased rate of uridine incorporation by resting CLL lymphocytes represented an inconstant observation, perhaps the result of variability between patients. However, the stimulation of uridine incorporation over resting levels was reproducible in each case. Thus, despite the diminished metabolic response at 48 hr, an initial PHA-induced augmentation of ³HU incorporation and the selective stimulation of rRNA labeling appeared to be intact in CLL lymphocytes.

Ribosomal RNA transcription and processing in CLL lymphocytes. For a more precise analysis of lymphocyte rRNA production, ³HMe was selected as the radioactive precursor. Of the entire cellular complement of mammalian cell RNA, only rRNA and transfer RNA (tRNA) are methylated (9). While in the nucleolus, newly transcribed 45S molecules become rapidly methylated. Cleavage of the 45S molecule into 32S and 18S products conserve methylated segments and only non-methylated segments are discarded (10). Subsequent cleavage of the 32S moiety also conserves the methylated segments in the 28S product (10). Therefore, the methylated segments of the 45S precursor molecule are quanti-

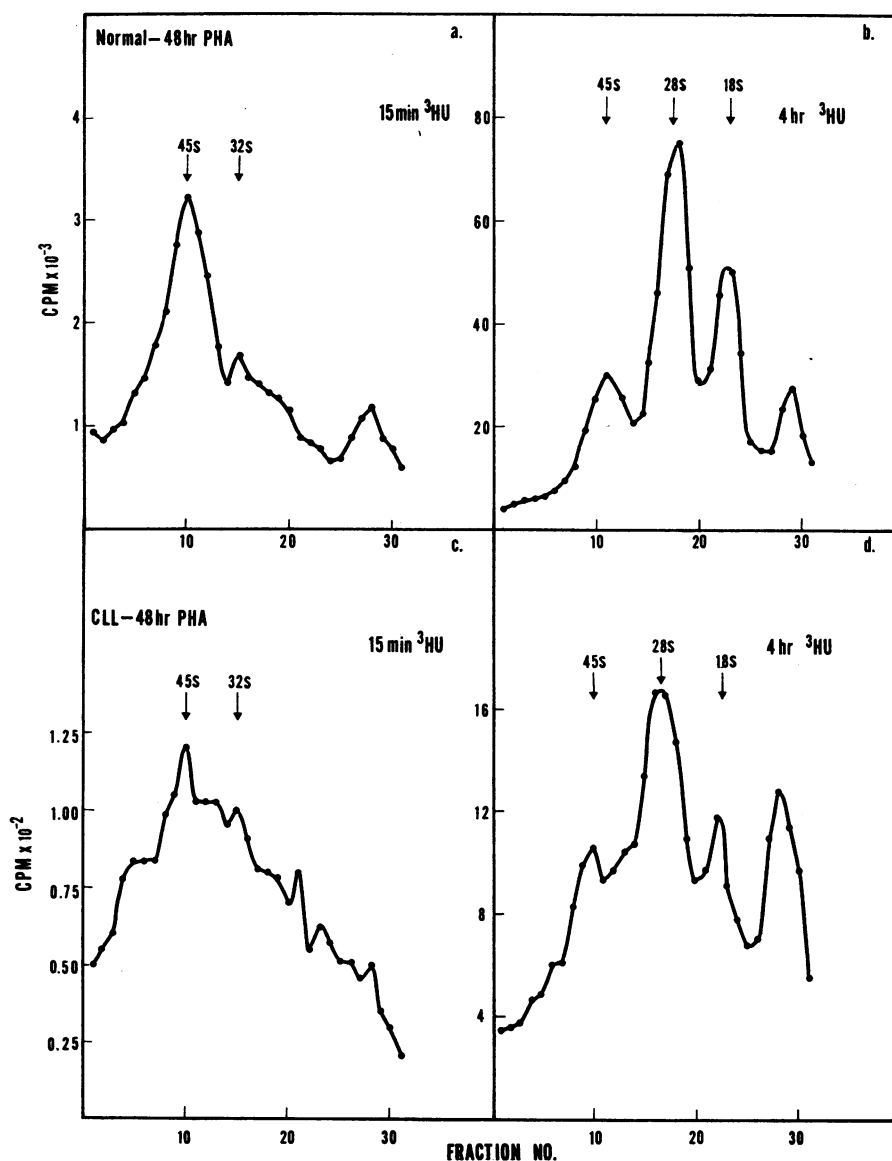


FIGURE 1 The pattern of ^3H -thymine incorporation into lymphocyte RNA. Prepared cultures from a single donor were incubated with PHA for 48 hr before exposure to a pulse of ^3H -thymine. Whole cell RNA was extracted and sedimented across sucrose gradients. Normal cultures: 15 min pulse (a), 4 hr pulse (b); CLL cultures: 15 min pulse (c), 4 hr pulse (d).

tatively retained in the mature 28S and 18S subunits of rRNA. This provided a convenient label for the various rRNA moieties.

Under the conditions of these experiments, the acid-soluble methyl donor pool in lymphocytes became saturated with radioactivity from ^3H -methyl groups in 1 hr (Fig. 3). Pretreatment of cultures with PHA for 1 hr did not increase the rate of ^3H -methyl incorporation into the acid-soluble pool and produced no effect on the rate of saturation. Both normal and CLL lymphocytes yielded identical re-

sults. The transient decrease in acid-soluble radioactivity probably represents adjustments in the pool size as labeled methyl groups are utilized for protein and *de novo* purine synthesis. From the shape of the curve it is apparent that the amount of methionine was not limiting.

In sedimentation profiles of radioactive methylated RNA, the rRNA moieties could be clearly defined and were not eclipsed by heterodisperse species which did not incorporate the methyl label. It was therefore possible to

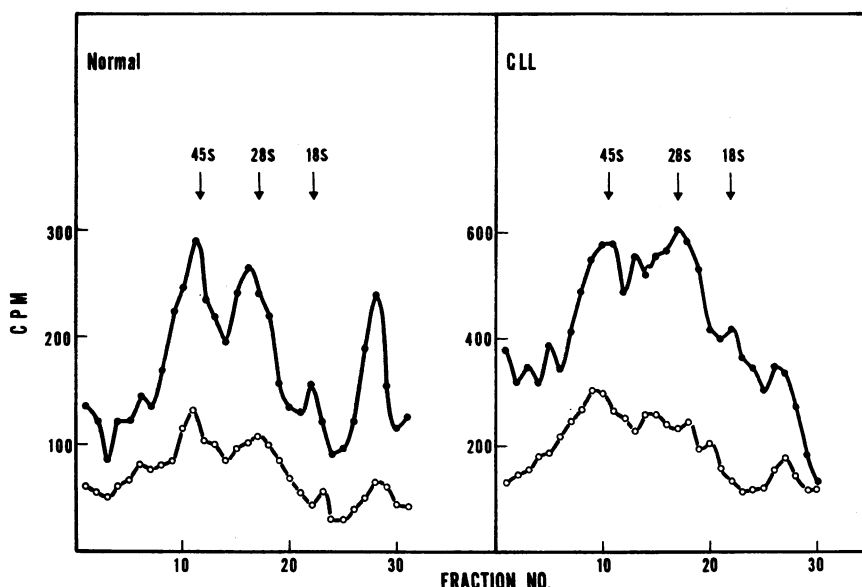


FIGURE 2 The pattern of $^3\text{H}\text{U}$ incorporation into lymphocyte RNA. After a 2 hr pulse of $^3\text{H}\text{U}$, whole cell RNA was extracted and sedimented across sucrose gradients. Resting cultures: (○—○); cultures incubated with PHA for 1 hr: (●—●).

use methylated RNA to directly follow the kinetics of 45S rRNA precursor transcription and processing to mature 28S and 18S ribosomal subunits.

Me RNA synthesized by normal resting lymphocytes during a 1 hr pulse of $^3\text{H}\text{Me}$ sedimented mostly in a discrete peak at 45S (Fig. 4a). Peaks at 32S-28S and particularly 18S assumed minor proportions illustrating the relatively slow rate at which nascent 45S RNA became cleaved and processed to mature rRNA subunits. 2 hr after the introduction of PHA these cells labeled Me RNA at approximately twice the resting rate (Fig. 4a). Furthermore, peaks of radioactive 28S and particularly 18S RNA became more prominent, suggesting an even greater PHA-induced increase in the processing of 45S rRNA precursor.

In a series of normal cultures derived from six individuals, the transcription rate of 45S RNA (Me RNA_{total}) increased by 80% during a 1 hr incubation with PHA (Fig. 5). The 45S transcription rate rose 13-fold over the first 48 hr in PHA-stimulated cultures of normal lymphocytes (Fig. 5). However, after 5 hr, there appeared to be no further increase in the rate of 45S processing as the relative prominence of 28S and 18S peaks with respect to the 45S peaks reached a maximum at 5 hr (Figs. 4a-c).

Cultures of resting CLL lymphocytes exhibited a pattern of rRNA synthesis which closely resembled the normal patterns as most of the nascent RNA methylated during a 1 hr pulse of $^3\text{H}\text{Me}$ sedimented at 45S (Fig. 4d). An over-all increase in radioactive methylated

RNA appeared in CLL cultures incubated with PHA for 2 hr (Fig. 4d). The augmentation in labeled RNA was most evident in the 28S peak with the 18S peak remain-

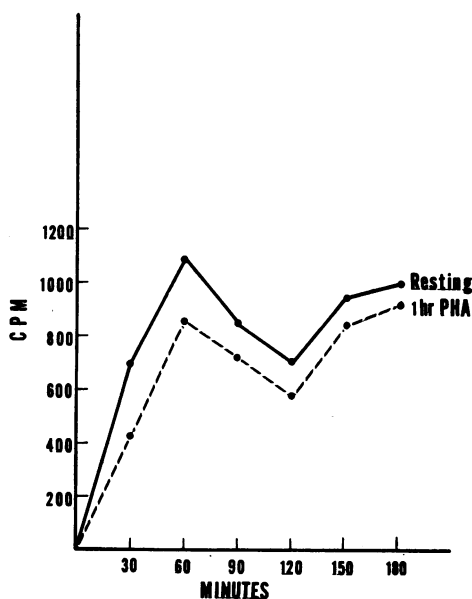


FIGURE 3 Saturation of an acid-soluble methyl donor pool in lymphocytes. Cultures were exposed to $^3\text{H}\text{Me}$ for varying periods of time before harvesting over a frozen slurry of Earles balanced salt solution. The washed cell pellet was then suspended in ice-cold 2% perchloric acid for 50 min. After centrifugation the supernatant was dissolved in a toluene-ethanol solution for liquid scintillation counting. Each point represents the mean of triplicate determinations.

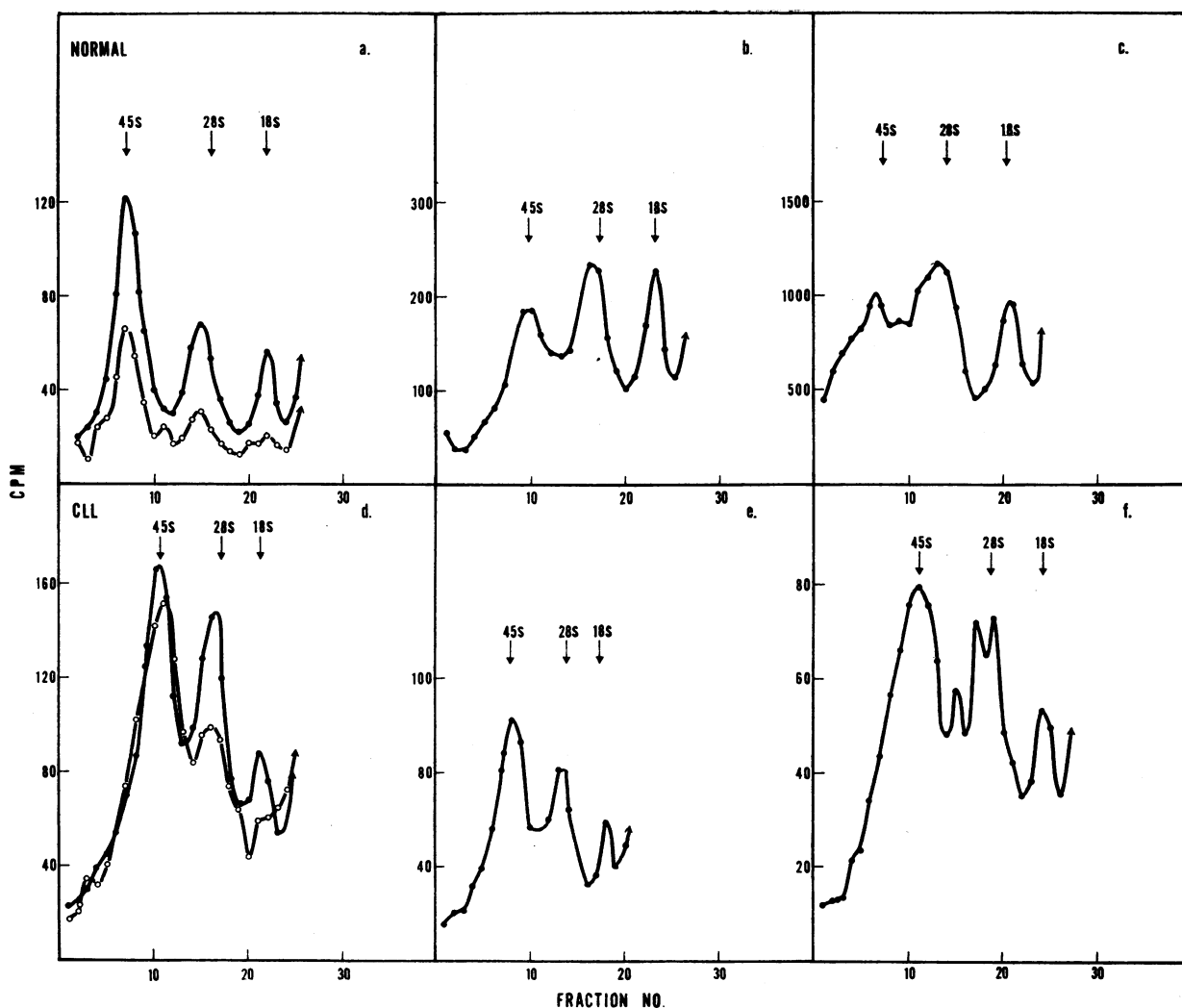


FIGURE 4 Sedimentation patterns of Me RNA. Lymphocyte cultures prepared from a single donor were exposed to ^3HMe for 1 hr before harvest. Whole cell RNA was extracted and sedimented across sucrose gradients. Resting culture: (\circ — \circ); PHA-treated cultures: (\bullet — \bullet); normal cultures: resting and 2 hr PHA (a), 5 hr PHA (b), 48 hr PHA (c); CLL cultures: resting and 2 hr PHA (d), 5 hr PHA (e), 48 hr PHA (f).

ing relatively less prominent. This latter observation can also be appreciated from the radioactive methylated RNA patterns in CLL cultures incubated with PHA for 5 and 48 hr (Figs. 4e and f). In these cultures, the over-all incorporation of label fell, but perhaps more significantly, the 45S, 28S, and 18S peaks retained their proportions.

Calculated values for augmented 45S transcription rates in CLL cultures stimulated with PHA for 1 hr (1.5 ± 0.3) approached the levels attained by normal cultures (1.8 ± 0.3) but continued incubation with PHA resulted in a rapid falloff towards resting levels by 5 hr (Fig. 5.) Therefore, when compared to normals, it appeared that the CLL cultures responded to PHA initially by an increased rate of 45S transcription and processing

to 28S but there was no selective rise in labeled 18S RNA. Furthermore, over the first 48 hr, the progressive increase in 45S RNA transcription failed to develop.

Cleavage of 45S rRNA precursor. Because of the complex, interrelated metabolic processes involved in the transcription and processing of rRNA, ratios calculated from the relative areas under the various peaks of sedimenting rRNA might not yield reliable information regarding the rates of 45S cleavage. Thus, nascent (labeled) 45S rRNA precursor molecules would enter a metabolic pool awaiting cleavage to 32S and 18S subunits. After the introduction of PHA, 45S transcription increased but as suggested by the prominent 28S peaks in Fig. 4b, the rate of cleavage seemed to increase to an

even greater extent. However, during cleavage, transcription would continue, probably at an increasing rate. Under these conditions, the 45S pool might approach saturation more quickly and therefore labeled 45S molecules might have been preferentially selected for cleavage thereby giving a more prominent 32S-28S peak relative to the 45S peak and a false impression of accelerated cleavage.

To directly measure the rate of 45S rRNA precursor cleavage before and after a 1 hr incubation with PHA, resting cultures and parallel cultures treated with PHA for 1 hr were allowed to incorporate label for 1 additional hr after which an aliquot was removed for RNA extraction and sedimentation analysis and the remaining cells were resuspended in an unlabeled medium. Aliquots were removed for RNA extraction and sedimentation analysis 30, 60, 90, and 120 min later. Mature rRNA was estimated from the sum of the areas under the 32S-28S peak (Me RNA_{32S-28S}) plus a calculated 18S peak (0.67 Me RNA_{32S-28S}). Me RNA_{total} was estimated in the usual fashion. Expressing Me RNA_{total} in the 1 hr pulse as 100%, labeled mature rRNA (Me RNA_{32S-28S} plus a calculated Me RNA 18S) was plotted as a negative log of the per cent of Me RNA_{total} against the time during which the cells were incubated in unlabeled medium. An example of such a

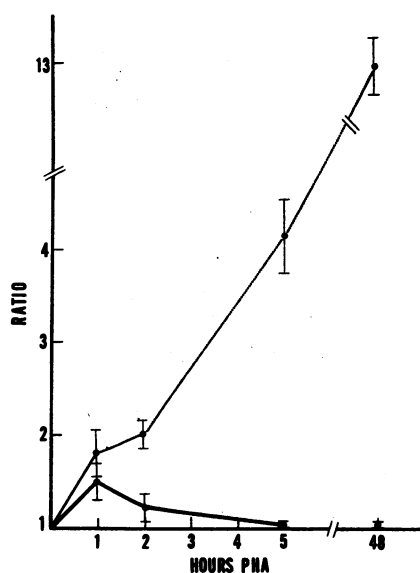


FIGURE 5 Augmentation of Me RNA_{total} in PHA-treated lymphocyte cultures. Cultures were incubated with PHA for specified intervals before exposure to a 1 hr pulse of ³HMe. Whole cell RNA was extracted and sedimented across sucrose gradients. The ratio was calculated from: (Me RNA_{total PHA})/(Me RNA_{total resting}). Each point represents the mean \pm 1 standard deviation of calculated R values obtained by analyzing lymphocyte cultures from six individual lymphocyte donors. Normal cultures: upper line; CLL cultures: lower line.

TABLE I

Rate* of Methylated 45S RNA Cleavage after a 1 hr Pulse†

	Resting	1 hr PHA	Per cent increase
Normal	0.35 \pm 0.03	0.53 \pm 0.04	51
CLL	0.34 \pm 0.03	0.50 \pm 0.04	47

* See Fig. 6.

† Expressed in arbitrary units representing six patients and six controls \pm 1SD.

curve is illustrated in Fig. 6. Identical curves were obtained when the data for the ordinate was derived from the disappearance of pulse-labeled Me RNA_{45S}, thus confirming a precursor-product relationship and demonstrating the quantitative conservation of 32S-28S subunits during the 2 hr interval. Furthermore, the logarithmic relationship suggested that processing of 45S precursor molecules involved first-order kinetics. In Fig. 6 neither line passed through the origin at time zero because the 1 hr pulse interval allowed sufficient time for processing of some 45S RNA.

The cleavage rate of nascent 45S rRNA precursor was estimated from the slope of the logarithmic function. In resting normal cultures, the curve yielded a slope of 0.35 and after a 1 hr incubation with PHA the slope increased to 0.53 (Table I). Thus, independent of the transcription rate, a 1 hr incubation with PHA resulted in nearly a 51% increase in the cleavage rate of 45S rRNA precursor. In similarly treated CLL cultures, the cleavage rate rose by 47%. Therefore, the initial reaction to PHA seemed to be nearly intact in CLL cultures. In the 1st hour after introduction to PHA, the rate of 45S transcription increased in CLL cultures as reflected in the total rRNA displayed in the sedimentation profile (Fig. 4), and, as in normal cultures, PHA initially produced an even greater increase in the rate of 45S cleavage.

Conservation of 18S RNA. The sedimentation patterns displayed in Fig. 4 suggested that, despite a PHA-induced increase in 45S rRNA precursor transcription and cleavage, CLL lymphocytes continued to waste 18S RNA for at least 48 hr. This phenomenon was expressed in quantitative terms by a series of pulse-chase experiments which allowed for processing of most of the nascent 45S RNA into discrete peaks sedimenting at 28S and at 18S. Thus, lymphocytes exposed to ³HMe for 1 hr were resuspended in an unlabeled medium for an additional hour during which approximately 80% of the labeled 45S RNA underwent initial cleavage. Planimetry yielded data to calculate relative values for $r = (\text{Me RNA}_{18S})/(\text{Me RNA}_{28S})$. Fig. 7a illustrates the phenomenon of wastage in resting normal lymphocytes while the early PHA-induced conservation appears in

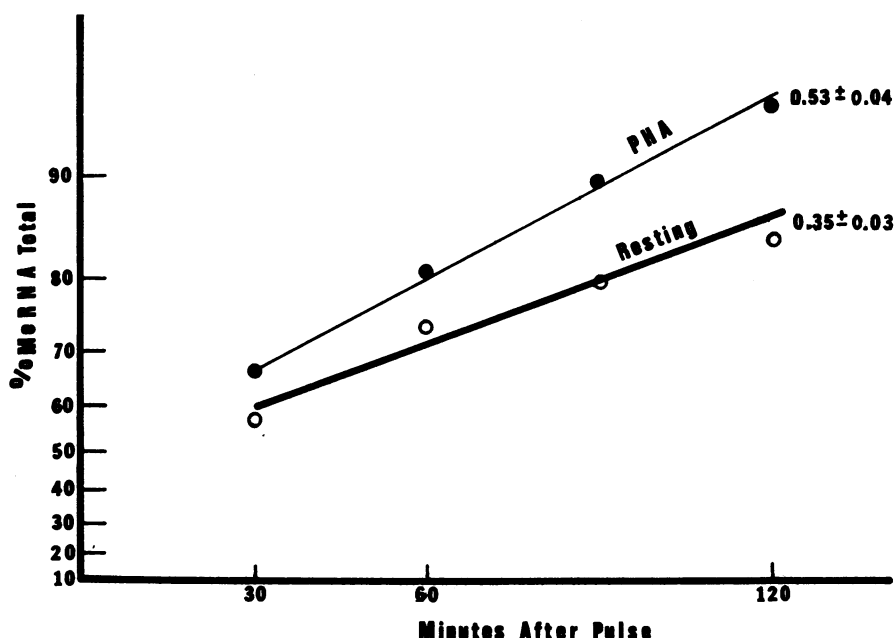


FIGURE 6 The rate of cleavage of 45S rRNA precursor in normal cultures. A pair of cultures (250×10^6 cells in 150 ml of medium) was exposed to a 1 hr pulse of ^3HMe . After removing an aliquot for RNA extraction and sedimentation analysis, the remainder of the cells was resuspended in unlabeled medium and the incubation continued. At specified times, aliquots were removed from RNA extraction and sedimentation analysis. Taking $\text{Me RNA}_{\text{total}}$ as 100%, the per cent of $\text{Me RNA}_{\text{total}}$ converted to $\text{Me RNA}_{28\text{S}-28\text{S}} + 0.67 \text{ Me RNA}_{28\text{S}-28\text{S}}$ was plotted on an inverted semilog scale against the hours incubated in the unlabeled medium. The slope of each line (i.e. the rate of $\text{Me RNA}_{4\text{S}}$ cleavage) was calculated from the change in the log of the percentage of $\text{Me RNA}_{\text{total}}$ converted to $\text{Me RNA}_{28\text{S}-28\text{S}} + 0.67 \text{ Me RNA}_{28\text{S}-28\text{S}}$ per unit time in the resting culture (O—O) and in culture treated with PHA for 1 hr (●—●). Number values included on graph represent the mean slopes ± 1 SD calculated from six separate experiments performed in an identical fashion (see Table I).

Fig. 7 *b*. CLL cultures similarly treated failed to exhibit 18S RNA conservation (Fig. 7 *d*, *e*). From Table II the comparative *r* values calculated from a series of sedimentation profiles clearly distinguished two types of PHA response. Conservation of 18S was nearly complete in normal cultures between 1 and 5 hr after the initial introduction of PHA where *r* values approached the ideal 0.67 (Table II). During the height of the growth response, at 48 hr, normal cultures once again

began to waste 18S RNA as *r* values fell to 0.55. In corresponding CLL cultures, no early PHA-induced conservation of 18S RNA appeared. In 48-hr cultures, the ratio showed a moderate increase.

As demonstrated in a previous publication (5), 168-hr cultures of PHA-treated normal lymphocytes, in the waning phase of their growth response, wasted considerable 18S RNA (Fig. 7 *c*). However, 168-hr cultures of PHA-treated CLL lymphocytes at the peak of their delayed response, showed efficient conservation of 18S RNA (Fig. 7 *f*). This observation demonstrated the association between 18S conservation and growth in CLL cultures.

The independent nature of the PHA-induced 18S conservation emerged from the following experiment. A pair of resting normal cultures was exposed to a 1 hr pulse of ^3HMe . Then the cells were resuspended in an unlabeled medium containing actinomycin D ($10 \mu\text{g}/\text{ml}$) and the incubation continued for an additional 2 hr chase. One of these cultures received PHA just before the 2 hr chase. Calculations of *r* (Fig. 8) yielded values

TABLE II
Conservation of 18S Subunits after PHA Stimulation

Lymphocyte culture	<i>r</i> ± 1 SD	
	Normal (6)	CLL (6)
Resting	0.38 ± 0.04	0.34 ± 0.07
1 hr PHA	0.63 ± 0.02	0.40 ± 0.06
5 hr PHA	0.64 ± 0.02	0.27 ± 0.10
48 hr PHA	0.55 ± 0.08	0.53 ± 0.06
168 hr PHA	0.48 ± 0.11	0.69 ± 0.10

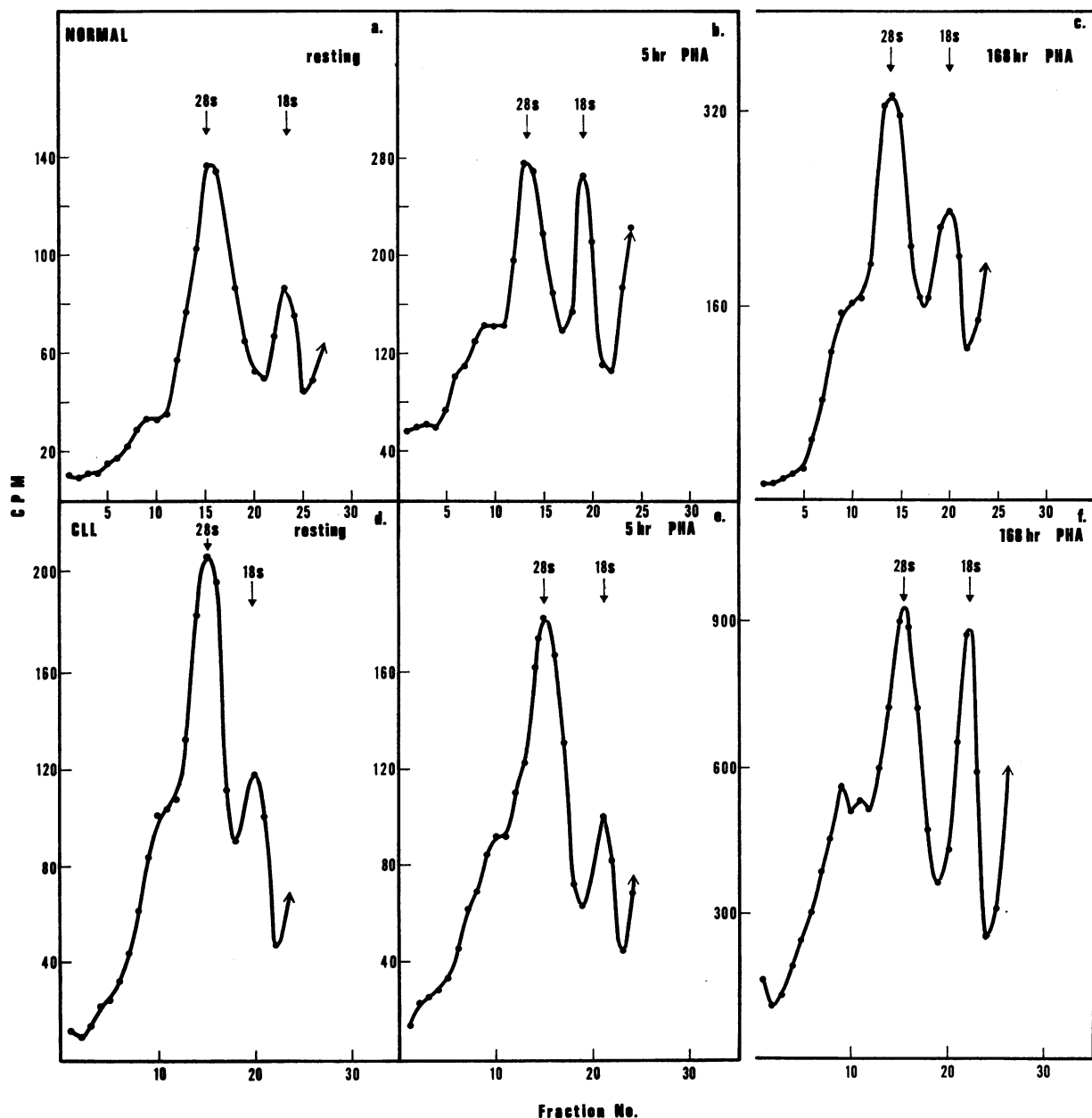


FIGURE 7 Sedimentation patterns of stable Me RNA in lymphocyte cultures. Each culture was exposed to a 1 hr pulse of ^3HMe after which the cells were resuspended in an unlabeled medium and incubated for an additional 1 hr chase. Whole cell RNA was extracted and sedimented across sucrose gradients. Normal cultures, resting (a); normal cultures, treated with PHA for 5 hr (b) and for 168 hr (c); CLL cultures, resting (d); CLL cultures treated with PHA for 5 hr (e) and for 168 hr (f).

of 0.35 in the untreated normal culture and 0.65 in the normal culture treated with PHA. Thus, PHA-induced conservation of 18S RNA, derived from labeled 45S precursor transcribed in the absence of PHA, indicated that PHA exerted its influence on 18S conservation

independent of its influence on increased 45S transcription.

CLL cultures similarly treated showed no conservation produced by PHA treatment (Fig. 8) confirming the previous observation that in CLL cultures PHA ex-

erted a stimulating effect on 45S transcription and cleavage without any accompanying inducement in 18S conservation.

DISCUSSION

Cultures of normal lymphocytes incubated with PHA for 48 hr contain 60–80% blast cells capable of proliferation, whereas in similarly treated CLL cultures

the blast cell count never rises above 10–15% (2). When compared to resting lymphocytes, PHA-treated cultures of proliferating lymphocytes rapidly incorporated ^3H U into RNA. However, in proliferating cultures, most of the stimulated uridine incorporation appeared in rRNA, thereby implying a selective increase in rRNA synthesis. The selective increase in rRNA labeling was detected after a 1 hr exposure to PHA. CLL lymphocytes ex-

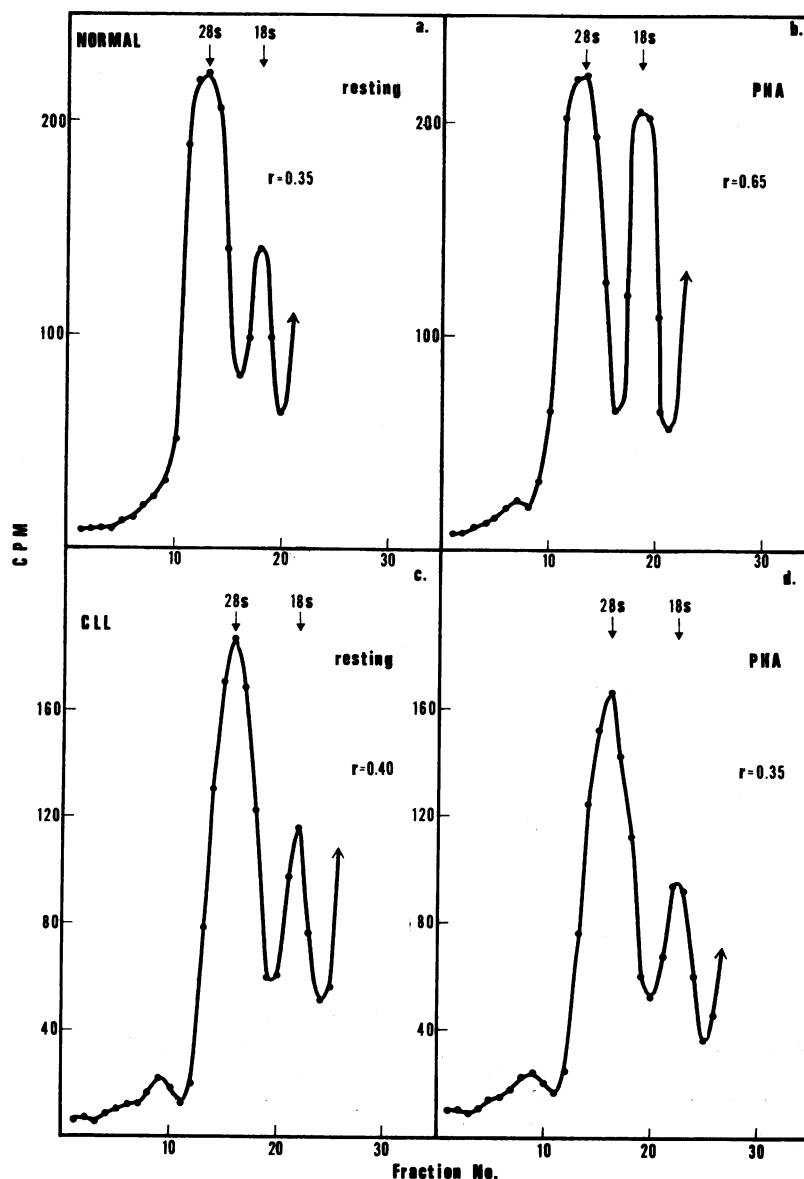


FIGURE 8 Fate of Me RNA under the influence of PHA. Resting cultures were exposed to a 1 hr pulse of ^3H Me and then resuspended in an unlabeled medium containing actinomycin D (10 $\mu\text{g}/\text{ml}$). One member of each pair was then treated with PHA (19 $\mu\text{g}/\text{ml}$). After a 2 hr additional incubation (chase), the cells were harvested and whole cell RNA was extracted and sedimented across sucrose gradients. Normal cultures, resting (a); normal cultures, PHA treated (b); CLL cultures, resting (c); CLL cultures, PHA treated (d).

posed to PHA for 1 hr also showed a selective increase in rRNA labeling, but at 48 hr, in the absence of cell enlargement, CLL lymphocytes resumed the resting pattern where most of the newly labeled RNA sedimented in a heterodisperse fashion. Therefore, the realization of a potential for growth in lymphocyte cultures seemed to be associated with the preferential synthesis of rRNA.

The possibility remains that heterodisperse RNA demonstrated on sedimentation profiles from CLL cultures represented partially degraded rRNA. However, the following observations render this unlikely: (a) optical density patterns of normal and CLL cultures were identical, thus eliminating the possibility of selective degradation of rRNA in CLL cultures; (b) specific activities of total acid-precipitable RNA and phenol-extracted RNA were identical so that no labeled RNA fraction was selectively lost from CLL lymphocytes during the extraction; and (c) the absence of heterodisperse methylated RNA in subsequent experiments demonstrated the nonribosomal nature of the heterodisperse moieties.

Employment of ^3HMe as a labeled precursor provided a means for quantitatively assessing the kinetics of rRNA transcription and processing. This technique was validated by the demonstration that the acid-soluble methionine precursor pool became saturated with label in 1 hr and that despite an increase in the rate of 45S labeling, the rate of pool saturation was unaffected by pretreating the cells with PHA. The usefulness of the methionine label takes on added significance considering the known scheme of rRNA synthesis where only the rRNA precursor portion of the 45S molecule (40% by weight) becomes methylated immediately after transcription in the nucleolus (10). During the series of cleavage steps, methylated segments of the original molecule are retained so that the concentration of methyl groups per specific rRNA moiety of known molecular weight can be accurately predicted. Cooper's calculation that the ideal value of 0.67 for the ratio $(\text{Me RNA}_{18\text{S}})/(\text{Me RNA}_{28\text{S}})$ presupposes that cleavage of each molecule of 45S rRNA precursor yields one molecule each of 28S and 18S RNA. Despite 18S wastage, Cooper found cytoplasmic rRNA always exhibited an ideal ratio (6). Such a finding might be anticipated since the cytoplasm contains mostly mature ribosomes which by necessity contain equal numbers of light and heavy subunits. Therefore, deviations from the ideal ratio probably result from unbalanced numbers of pre-ribosomal particles in the nucleolus. Furthermore, the imbalance must result from intranucleolar degradation of the 18S subunit (or its precursor). Since the resting lymphocytes do not accumulate 32S-28S RNA, eventual degradation of unbalanced 32S-28S moieties must also take place, but at a slower rate than the corresponding 18S RNA degradation.

Our calculations of 45S transcription assumed minimal wastage of 32S moieties during the interval under analysis. Studies of the kinetics of 45S processing confirmed this assumption. Thus, following a pulse of ^3HMe total 45S transcription was estimated from the formula: $\text{Me RNA}_{\text{total}} = \text{Me RNA}_{45\text{S}} + \text{Me RNA}_{32\text{S}-28\text{S}} + 0.67 \text{ Me RNA}_{32\text{S}-28\text{S}}$. An expression for the rate of 45S cleavage, independent of the transcription rate, was estimated from the slope of the line presenting the first-order kinetic equation for 45S RNA precursor processing to 32S-28S and 18S products. Finally, conservation of 18S RNA independent of transcription and initial cleavage was calculated directly from the $(\text{Me RNA}_{18\text{S}})/(\text{Me RNA}_{28\text{S}})$ ratios after nearly complete processing of labeled 45S precursor. Standard deviations obtained from the pooled data of repetitive experiments eliminated the uncertainties resulting from the imprecision of the planimetry technique.

The rate of 45S transcription doubled in normal lymphocytes after a 1 hr incubation with PHA and then continued to rise to 13 times the resting rate by 48 hr when these cultures contained many proliferating blast cells. In PHA-treated CLL cultures, an initial rise in the rate of 45S RNA transcription was detected at 1 hr after which 45S RNA transcription fell off to base line levels at 48 hr (when CLL cultures contained few blast cells). Therefore, PHA-induced blast transformation was associated with a progressive rise in the rate of 45S RNA transcription.

Increased rates of 45S processing could be detected in normal cultures treated with PHA for 1 hr. This indicated that, in addition to an increased rate of transcription, PHA induced an even more rapid cleavage of each newly transcribed 45S molecule to 32S and 18S products. This early phenomenon was also present in CLL cultures treated with PHA for 1 hr. Therefore, the initial effects of PHA on transcription and processing of 45S RNA remained intact in CLL lymphocytes. Nevertheless, the initial response of PHA-treated CLL lymphocytes remained abnormal in that conservation of 18S RNA failed to develop. Only at 168 hr did PHA-treated CLL lymphocytes begin to conserve 18S RNA.

The actual role of 18S RNA conservation in the initiation of normal lymphocyte growth presents an intriguing problem. PHA exerted an effect on 18S RNA conservation independent of stimulated 45S RNA transcription as rRNA transcribed by resting cells was also conserved under the subsequent influence of PHA. Furthermore, maximal conservation in PHA-treated normal cultures occurred within the first 5-20 hr. Wastage of 18S RNA resumed during the most active phase of 45S transcription and cell growth. These findings implied that 18S conservation may exert a direct effect on the initiation of lymphocyte growth. In the PHA-treated

CLL cells, failure to conserve 18S RNA may have aborted the independent PHA-induced stimulation of 45S RNA transcription and processing.

The requirement for maximal 18S RNA conservation on the part of PHA-treated CLL cultures at 168 hr contrasted sharply with the partial wastage seen in 48-hr cultures of PHA-treated normal lymphocytes (Fig. 7). 48 hr represents the height of the growth response to normal cultures while CLL cultures would be growing maximally at 168 hr (2, 3). Thus, the delayed onset of cell growth seemed to be closely associated with 18S RNA conservation in CLL cultures. This observation further supports the contention that 18S RNA conservation plays a role in facilitating increased synthetic activity necessary for cell growth and points to the possibly unique nature of the CLL lymphocyte which apparently continues to utilize all of its rRNA precursor even during the phase of active growth.

Resting lymphocytes contain very few cytoplasmic ribosomes when compared to the dense clusters of these organelles in proliferating blast cells (11). Conceivably the initiation and maintenance of the growth process in lymphocytes requires the delivery of new cytoplasmic ribosomes at a sufficient rate to support increased synthesis of enzymes and structural proteins. Therefore, maintenance of the resting state might depend on a restriction of ribosome synthesis below this critical rate. This would provide for turnover with no net accumulation of ribosomes. Restriction of ribosome synthesis could be controlled at the level of 45S RNA transcription and also at the level of utilization of transcribed 45S rRNA precursor. Evidence derived from the present data indicated that, as a result of 18S RNA wastage, about one-half of the newly transcribed rRNA precursor molecules was not utilized for ribosome assembly by resting lymphocytes. Under these circumstances, over-all RNA and protein synthesis remained at low levels and the cells did not enlarge. Within 1 hr after introduction of PHA, the initial increase in 45S RNA transcription was effectively multiplied by a greatly increased efficiency of utilization of newly formed precursor molecules for ribosome assembly. Increased protein synthesis would be a likely consequence of increased ribosome concentration in the cytoplasm. This burst of new protein synthesis could then set the stage for heightened nuclear metabolism, in an escalating fashion, leading to progressively more active RNA and protein synthesis which would ultimately culminate in cell enlargement.

During the phase of active cell enlargement the rate of RNA synthesis might have reached sufficient proportions such that net ribosome synthesis could proceed despite inefficient utilization of newly transcribed rRNA precursor. Furthermore, the resumption of 18S RNA wastage might begin to limit the growth rate by slowing

the rate of new ribosome delivery and thereby set the stage for retransformation to the resting state.

Despite the initial increase in transcription and processing, continued wastage of 18S RNA still precluded the utilization of one-half of the newly formed precursor molecules in PHA-stimulated CLL lymphocytes. Conceivably this situation restricted the rate of new ribosome delivery below the critical level and prevented the subsequent escalation of protein and RNA synthesis until the ultimate development of efficient 18S RNA conservation.

The independent nature of the PHA-induced conservation of 18S RNA in normal lymphocytes and its selective absence in CLL lymphocytes raises the possibility that CLL lymphocytes may harbor a defect which renders these cells relatively insensitive to the growth-promoting influence of PHA. Such a phenomenon serves to emphasize the probable importance of mechanisms governing 18S RNA conservation in the initiation of lymphocyte growth and the role of 18S RNA conservation in the eventual delayed growth of PHA-treated CLL lymphocytes.

While the primary site of PHA action remains in dispute (12), accumulating evidence has implicated the cell membrane as the primary PHA receptor (13, 14). Subsequent PHA-induced effects on nuclear metabolism may result secondarily from events initiated at the cell surface. However, there may be more than one PHA-activated membrane mechanism, not the least important of which may secondarily influence 18S RNA conservation in the nucleolus. A demonstrated requirement for active protein synthesis in continuous ribosome formation in lymphocytes (15) might reflect an interaction between newly synthesized protein and the 18S RNA precursor in the nucleolus. Such an intermediate protein formed at the cell surface upon contact with PHA could then traverse the cytoplasm and appear in the nucleolus to protect against 18S RNA degradation. Reduced PHA receptor sites have been observed in CLL lymphocytes (14). Perhaps a lack of sufficient receptor sites to support the initial conservation of 18S RNA represents a specific defect which renders CLL lymphocytes incapable of a prompt response to PHA.

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