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Research Article

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Characteristics of the RNA Synthesized in Vitro by the Normal Human Small Lymphocyte and the Changes Induced by Phytohemagglutinin Stimulation

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ABSTRACT These studies demonstrate that the circulating human small lymphocyte synthesizes ribonucleic acid (RNA) of high molecular weight which is not primarily a ribosomal precursor and which is, in part, complementary to human deoxyribonucleic acid (DNA). The stimulation of these lymphocytes by PHA brings a cell population in which few ribosomes are synthesized to a functional condition in which a large amount of these particles are produced. This increase in the synthesis of ribosomal RNA is one of the earliest and most relevant effects of PHA on the RNA metabolism of small lymphocytes.

INTRODUCTION

The small lymphocyte has been regarded as a completely differentiated cell representing the end stage of lymphoid maturation. This opinion has received support from many experimental observations including the lack of deoxyribonucleic acid (DNA) synthesis in these cells (1), their low rate of protein synthesis (2, 3), and their long life span (4, 5). On the other hand, the central position of the circulating small lymphocyte in both cellular (6) and humoral (7) immune reactions has been firmly established. Moreover, it has been demonstrated that these cells have the ability to grow and divide in vitro under the action of phytohemagglutinin [PHA (8, 9)], as well as several other substances. The rapidly growing knowledge about the "reactivity" of the small lymphocyte has raised interest about the regulation of protein synthesis in these cells. Knowledge of the macromolecular metabolism of their RNA is a necessary prerequisite for a basic understanding of such regulation.

The metabolism of RNA has been previously investigated in several types of mammalian cells, and two different patterns have emerged. The first one has been observed mainly in proliferating tissue culture cells, such as the HeLa, L, or FL types. In these cells, the newly synthesized nuclear RNA appears to consist of a polydisperse "giant" RNA [60–80S (10)], whose function is not well defined, and a 45S ribosomal precursor molecule (11, 12). This 45S ribosomal precursor RNA is rapidly split to a 35 and an 18S molecule, the latter being immediately transferred from the nucleus to the cytoplasm. The 35S RNA is in turn cleaved to a 28S ribosomal RNA which then enters the cytoplasm (13). This sequential pattern was recently confirmed by the study of the methylation of RNA in the HeLa cell (14). It had been previously demonstrated that only ribosomal and transfer RNA in bacterial and animal cells contained methylated bases and sugar moities (15–19). The methylation of ribosomal RNA, whose methyl groups are found almost entirely on ribose moieties (14, 15), occurs in the nucleolus at the

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level of the 45S ribosomal precursor (14, 20). This reaction is completely suppressed by the action of actinomycin D (20, 21), which suggests that the process proceeds only in the presence of a continuing supply of "nascent" RNA. Since the polydisperse nuclear RNA of HeLa cells is not methylated, it was possible with this radioactive label to follow more precisely the various steps in the formation of mature ribosomal RNA (14).

The second pattern of mammalian RNA metabolism has been described recently in mature duck erythroblasts (22, 23) and in small lymphocytes of chronic lymphocytic leukemia [CLL (24)]. In these highly differentiated nonproliferating cells the bulk of the newly synthesized RNA has a high sedimentation coefficient (30–80S) but is converted at a very low rate, if at all, to 28 and 18S ribosomal RNA. In the mature avian erythroblast the base composition of the 30–80S RNA labeled for short intervals [15–45 min (23)] is definitely different from that of ribosomal RNA and more closely resembles that of DNA. In CLL lymphocytes (24) the RNA pulse-labeled for 1 hr had a base composition similar to that of ribosomal RNA; however, it formed DNA-RNA hybrids to the extent of 19–30%. This occurrence indicates that a substantial part of this RNA consisted of a heterogenous mixture of sequences of nucleotides complementary to DNA. The CLL lymphocytes are functionally impaired, most of them being unable to transform under the action of PHA (25). Therefore, no inference could be drawn from these studies about the RNA metabolism of the normal small lymphocyte. In the work reported here an attempt has been made to determine some of the characteristics of the rapidly labeled RNA synthesized in vitro in circulating human lymphocytes by studying its sedimentation properties in sucrose gradients, its methylation, and its ability to form RNA-DNA hybrids with homologous DNA. Cells were studied both in the resting state and within 24 hr after phytohemagglutinin stimulation.

METHODS

Preparation of lymphocytes. Leukocyte-enriched plasma was obtained from heparinized blood (10,000 U/500 ml of blood) of normal volunteers after the red cells were sedimented by gravity at 37°C in the presence of 0.6% dextran (Abbott Laboratories, North Chicago, Ill.). Lymphocytes were separated by a modification of the method of Greenwalt, Gajewski, and McKenna (26). The supernatant plasma was diluted with 1 volume of warm minimal essential medium (MEM) containing penicillin and streptomycin and the suspension filtered slowly at 37°C through a column of commercial nylon fibers (Fenwal Leukopak, Fenwal Laboratories, Morton Grove, Ill.), 25 × 150 mm. Before use the column was washed for 1 hr with distilled water, dried by suction, and autoclaved. The column was rinsed with 1 volume of warm MEM, and the final cell suspension was centrifuged at 1200 rpm for 15 min. The supernatant was discarded, and the cell pellet was suspended in a small volume of autologous plasma and counted. Finally, the cells were diluted with MEM and 20% autologous plasma to a concentration of 2–3 × 10^6 cells per ml. The average yield was 4–6 × 10^8 lymphocytes per 500 ml of blood. All operations were performed under sterile conditions.

Time course studies. For kinetics experiments, 100-ml cultures were set up in 8-oz prescription bottles. Uridine-5-^{3}H (30.0 c/mmole, Nuclear-Chicago Corporation, Des Plaines, Ill.) and phytohemagglutinin (Burroughs-Wellcome & Co., Tuckahoe, N. Y.), or uridine-^{3}H alone, were then added, and duplicate 10-ml aliquots were taken at different intervals. The content of each vial of phytohemagglutinin was dissolved in 5 ml of water and used at concentration of 0.02 ml/ml. The cells were harvested by chilling on frozen saline and centrifugation at 1200 rpm and washed once with ice-cold saline. Analysis by the Schmidt-Thannhäuser procedure (27) revealed that virtually no uridine-5-^{3}H was incorporated into DNA. RNA was assayed by a modification of the Schneider procedure (28). The cell pellet was resuspended in 2 ml of 10% trichloroacetic acid (TCA) at 4°C for 30 min, centrifuged, the precipitate washed twice with cold 5% TCA and once each with 80% ethanol, 95% ethanol, and ethanol-ether 1:1. The dried residue was then hydrolyzed at 90°C for 15 min in 2 ml of 5% TCA and centrifuged. The supernatant, pooled with two TCA washings, was used for radioactivity determinations.

Labeling of cellular RNA for sucrose gradient analysis and RNA-DNA homology studies. For RNA sedimentation studies, the lymphocyte suspension was divided into 20-ml cultures in 2-oz prescription bottles that were left overnight at 37°C. Incubations were started by adding the different labeled precursors, with or without previous PHA treatment, and harvesting the cells at different intervals, as specified in the Results section. In all sedimentation studies, because of the low number of lymphocytes available for each experiment, unlabeled RNA from KB cells (human epidermoid carcinoma) was used as carrier. These cells were obtained from Microbiological Associates, Bethesda, Md. and maintained at cell counts of 100,000–400,000 cells/ml in spinner flasks with Eagle's medium (29) supplemented with 5% horse serum, glutamine 0.3 g/ml, penicillin, and streptomycin. 6–9 × 10^7 lymphocytes were extracted together with 1–2 × 10^8 KB cells in each experiment. For DNA–RNA homology studies, unstimulated and PHA-treated lymphocytes were maintained in 100-ml cultures in 8-oz prescription bottles. Several bottles of labeled or unlabeled cells were pooled

and harvested in order to obtain the relatively large amounts of RNA necessary for "hybridization" and "competition" experiments.

RNA and DNA extraction procedures. For sucrose gradient analysis the RNA was extracted by a modification of the methods of Scherrer and Darnell (11) and Kirby (30). The cell pellet was thoroughly resuspended in 20 ml of 0.01 M sodium acetate buffer, pH 5.2, with 0.01 M sodium ethylenediaminetetraacetate (NaEDTA) and 0.05% bentonite (31). Sodium dodecyl sulfate (SDS) was added to the final concentration of 0.5% and the cells shaken vigorously by hand at room temperature for 30 sec. 20 ml of a 90% solution of phenol–*m*-cresol (7.9 : 1 v/v) containing 0.1% 8-hydroxyquinolone was then added and the material shaken in a water bath at 60°C for 3 min. The suspension was cooled rapidly in a dry ice-alcohol mixture and centrifuged for 10 min at 11,000 rpm in a Sorvall RC-2, Ivan Sorvall Inc., Norwalk, Conn., at 4°C. The aqueous phase was reextracted with hot phenol–*m*-cresol, containing 0.5% SDS, and the RNA precipitated from the aqueous phase by the addition of 2 volumes of ethanol–*m*-cresol (9 : 1, v/v) and 1/10 volume of 20% potassium acetate (KAc), pH 5.4. After standing at −30°C for at least 1 hr the precipitate was collected by centrifugation, washed once with 70% ethanol containing 2% KAc, and redissolved in 0.01 M Tris at pH 7.4.

For hybridization experiments, the RNA extraction procedure was modified in order to minimize DNA contaminations. Because of the high DNA-RNA ratio in these cells, when a large pellet was extracted with SDS-phenol, much of the RNA was trapped in the sticky mass of DNA. Therefore, 1 ml of high ionic strength buffer (0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris, pH 7.4) and 1 mg of electrophoretically purified deoxyribonuclease [DNase (Worthington Biochemical Corp. Freehold, N. J.)] were added to the pellet and the mixture stirred on a Vortex mixer, (The Vortex Mfg. Co., Cleveland, Ohio) until it was no longer viscous and there were no visible clumps (13). 2 min of treatment with DNase was usually sufficient. The preparation was diluted with 20 volumes of 0.01 M NaAc buffer, pH 5.2, and with 0.01 M NaEDTA and 0.05% bentonite, and the phenol extraction carried out as previously described. After the initial ethanol–*m*-cresol precipitation the precipitate was washed twice with cold 3 M NaAc, pH 6 (30), and dissolved in 5 ml of 0.01 M Tris, pH 7.4. 200 μg of DNase with 10 μl of 1.0 M MgAc were added, and the solution was incubated for 10 min at 37°C. 50 μg of subtilisin (Nagarse, Enzyme Development Corp., New York) was then added and incubated for 5 min. The reaction was stopped with 0.5 ml of 1% SDS and 0.1 ml of 1.0 M NaEDTA. NaCl was added (final concentration 0.15 mole/liter, the solution was extracted with an equal volume of hot phenol–*m*-cresol, and the RNA precipitated from the aqueous phase with 2 volumes of ethanol. The RNA pellet was collected by centrifugation and dissolved in 2 × SSC[1] and 0.05% SDS.

[1] SSC refers to 0.15 M NaCl and 0.015 M sodium citrate, pH 6.7.

DNA was extracted from normal lymphocytes and mouse lymphocytes (L1210 leukemia) by the Marmur method (32).

Sucrose gradient analysis. 5–20% linear sucrose gradients were prepared in 0.01 M Tris buffer, pH 7.4, with 0.1 M NaCl. 15–25 OD units of RNA in a volume of 0.5 ml or less were layered on the top of each gradient. Centrifugations were carried out at speeds from 16,000 to 20,000 rpm in the SW 25.1 rotor of the Spinco Model L ultracentrifuge for 16 hr at 4°C. Fractions of 0.6 ml were obtained with the use of a gradient analyzer (Union Carbide Corp., Oak Ridge, Tenn.) for optical density and radioactivity determinations.

Nucleic acid homology studies. The ability of labeled lymphocyte RNA to form hybrids in vitro with unlabeled DNA was investigated by a modification of the membrane filter method of Nygaard and Hall (33).

Suitable amounts of ^{3}H-labeled RNA were reacted with varying amounts of lymphocyte DNA in a total volume of 0.1 ml of 2 × SSC and 0.05% SDS in glass ampules. In competition experiments, radioactive RNA was diluted with the unlabeled RNA before it was placed in the ampules. The amount of radioactive RNA used in each experiment was enough to approximately saturate the readily available sites of a designated amount of DNA, and this value was obtained from the saturation curve for each RNA sample. It was assumed that when the radioactive RNA was diluted with an equal amount of unlabeled homologous RNA the amount of radioactive RNA recovered as a hybrid would decrease by 50%. Further dilution of the labeled RNA with increasing amounts of unlabeled RNA would allow one to construct a theoretical competition curve and to compare it with actual values. A decrease of radioactivity in the hybrid is assumed to be specific and to result from competition between the labeled and unlabeled RNAs for the same sites on the DNA. When low levels (less than 150 μg of RNA per vial) of unlabeled RNA were used it was possible to perform competition studies which followed the theoretical curve. This maneuver is both practical, as far as amounts of RNA needed for a given experiment, and avoids the problem of nonspecific interference that may result in experiments with very high RNA–DNA ratios (34, 35). The sealed ampules were heated to 100°C for 15 min, cooled rapidly in ice water, and incubated for 16 hr at 67°C. The content of each ampule was diluted in 15 ml of filtration buffer (0.01 M Tris, 0.5 M NaCl, 0.01 M $Na_4P_2O_7$, pH 7.3) and filtered through membrane filters (Millipore HAWP, 45 mm, Millipore Filter Corp., Bedford, Mass.) which had been previously soaked in the filtration buffer overnight. The filters were washed with 200 ml of 60°C filtration buffer, placed into petri dishes, and covered with 10 ml of a heat-treated RNase solution [(32) 20 μg/ml in 2 × SSC, Sigma Chemical Co., St. Louis, Mo.]. After 90 min the reaction was stopped by the addition of 0.5 ml of 1% SDS, the filters were washed again with 200 ml of 60°C filtration buffer, with a final wash of 25 ml of 95% ethanol, and dried at 70°C.

Radioactivity assay procedures. In kinetics experiments, aliquots of the TCA extracts were mixed directly with a

dioxane-containing phosphor (36) and counted in a Packard Tri-Carb liquid scintillation spectrometer. Radioactive RNA in sucrose gradient fractions was precipitated by addition of 100 μg of bovine serum albumin and an equal volume of 10% TCA. After standing at 4°C for at least 10 min the precipitate was collected on Millipore filters (AAWP, 25 mm), and washed twice with cold 5% TCA. The dried filters were counted in a toluene-phosphor counting solution. In the hybridization experiments the radioactivity on the filters was assayed in a three channel liquid scintillation spectrometer (Nuclear-Chicago Corp; Des Plaines, Ill.), and the channels ratio method was used to correct for variable quenching. Samples were counted for a sufficiently long time to obtain statistical accuracy of ± 3% except for the methyl-^{14}C-methionine experiments in which the accuracy was ± 6% or better.

RESULTS

Characteristics of the cell population studied. Giemsa stained smears showed that the cell population prepared by separation on nylon fiber columns consisted almost exclusively of small and medium lymphocytes. Less than 1% of the cells were large lymphocytes, and less than 0.5% were granulocytes. The "stimulation" by PHA was measured by evaluating the number of "transformed" blastic cells present in the cultures after 48 hr of incubation with PHA. The criteria for morphological identification of the cells in the intermediate stages of transformation and of the fully developed "blasts" were those decribed by Quaglino, Hayhoe, and Flemans (37). In most experiments the percentage of transformed cells at 48 hr was greater than 50%. When this was not true the data were discarded.

Kinetics of uridine-^{3}H incorporation by resting and PHA-stimulated lymphocytes and metabolic stability of the newly synthesized RNA. Normal peripheral lymphocytes maintained in vitro without addition of PHA incorporated tritiated uridine into acid insoluble material for at least 40 hr. In contrast to previously reported studies (38, 39), we did not observe a decreased incorporation of tritiated precursors into RNA of normal lymphocytes during the first 24 hr in culture. In PHA-treated lymphocytes the uptake of precursors after 2 hr of exposure to PHA was twofold greater than in unstimulated cells, and by 24 hr the uptake of uridine-^{3}H into acid insoluble material was 12-fold greater in these cells when compared to control lymphocytes. This observation merely confirms what has been previously reported from several laboratories (38–41). The stability of the labeled RNA was evaluated by removing 20-ml samples into a separate flask and incubating them with actinomycin D (2.5 μg/ml) for 2 hr. It was found in both unstimulated and stimulated cultures that the percentage of the radioactive material which became acid soluble after addition of the antibiotic was approximately 30% at 1, 4, 8, and 12 hr and approximately 45% at 24 hr.

Sedimentation pattern of labeled RNA after incubation with uridine-^{3}H. Fig. 1 shows the sedimentation pattern of whole cell RNA extracted from normal unstimulated lymphocytes after exposure to uridine-^{3}H for 1 and 4 hr. About 90% of the radioactivity incorporated during 1 hr of exposure to the isotope sedimented in the 30–60S region of the gradient (Fig. 1 a). The amount of ribosomal RNA labeled did not appear significantly increased after 4 hr of incubation with the isotope (Fig. 1 b). Even after 12 hr of incubation more than 75% of the radioactivity sedimented in regions of the gradient corresponding to S values higher than 30. Only after 24 hr of exposure to the labeled precursor did the percentage of radioactivity associated with 28 and 18S ribosomal RNA definitely exceed that in heavier RNA.

The sedimentation pattern of 1 hr pulse-labeled RNA from lymphocytes preincubated for 1 hr with PHA are shown in Fig. 2 a. The pattern was not significantly different from that in unstimulated cells since most of the radioactivity (80%) sedimented with an S value higher than 30. The difference between these data and those of a previously reported study, in which the synthesis of a polydisperse (4–30S) RNA was reported (42), may be related to improvements in the technique for extraction of mammalian RNA. In fact, the same author, using the RNA extraction procedure described in this paper, has now demonstrated results comparable to those recorded here (43). After 3 further hr of incubation with the tritiated precursor the sedimentation pattern was quite different (Fig. 2 b). Three definite radioactive peaks were observed in coincidence with the optical density peaks of 28, 18, and 4S RNA, although 60% of the label sedimented with heavier RNA. An even higher rate of labeling of ribosomal RNA was found in lymphocytes preincubated for 6 hr with PHA. Under these conditions the percentage of radioactivity sedimenting with an S value

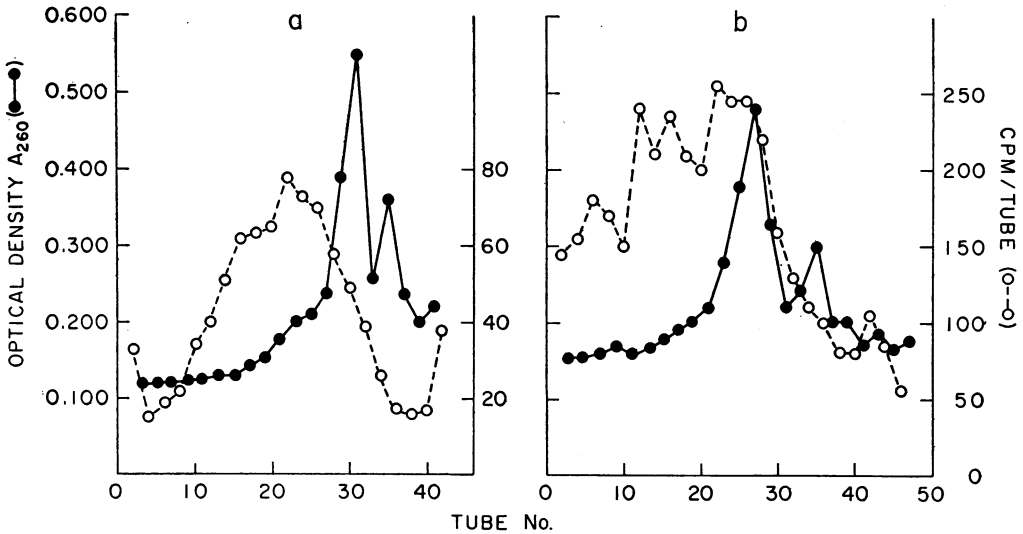

FIGURE 1 Sedimentation patterns of labeled RNA extracted from unstimulated lymphocytes incubated with uridine-^{3}H (10 μc/ml) for 1 (*a*) and 4 (*b*) hr. Since in all sedimentation studies labeled lymphocyte RNA and unlabeled KB cell RNA were extracted simultaneously, the optical density profile (●—●) in this and in the following figures represents mainly the unlabeled KB RNA. Gradients were centrifuged at 17,000 rpm for 16 hr at 4°C in two different centrifuges.

greater than 30 was 75% after 1 hr of incubation with uridine-^{3}H (Fig. 3 a) but only 28% after 4 hr of exposure to the label (Fig. 3 b).

Sedimentation analysis of uridine-^{3}H-labeled RNA after actinomycin chase. The inhibitory action of actinomycin D on DNA-dependent RNA synthesis was utilized to investigate the fate of the rapidly labeled RNA. The sedimentation pattern of labeled RNA extracted from cells incubated for 1 hr with the labeled precursor was compared with that of RNA extracted from cells labeled for the same period of time followed by a 3 hr incubation with actinomycin D. RNA extracted from cells labeled for 4 hr without actinomycin was also analyzed.

The experiment was performed at the same time on two sets of lymphocyte cultures. In one of them, unstimulated cells were incubated with uridine-2-C^{14} (37.0 mc/mmole, Nuclear-Chicago Corp., Des Plaines, Ill.). In the other, PHA was

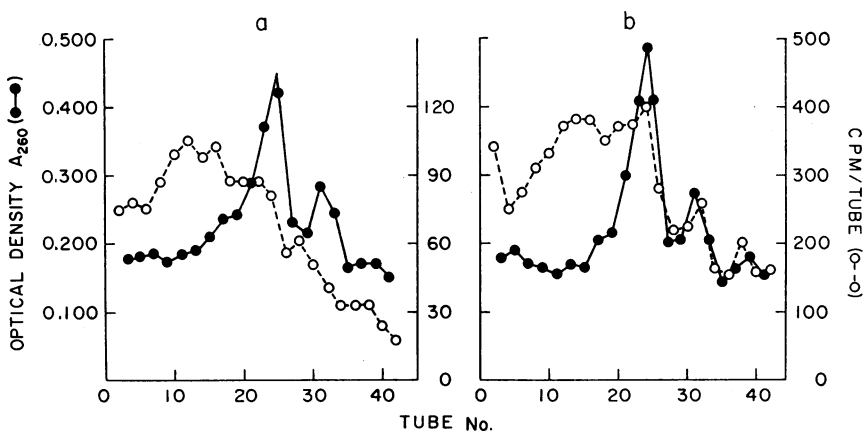

FIGURE 2 Sedimentation patterns of labeled RNA from PHA lymphocytes incubated with uridine-^{3}H (10 μc/ml) for 1 (*a*) and 4 (*b*) hr. Uridine-^{3}H was added to the cultures after 1 hr of preincubation with PHA. Gradients were centrifuged at 17,000 rpm for 16 hr at 4°C.

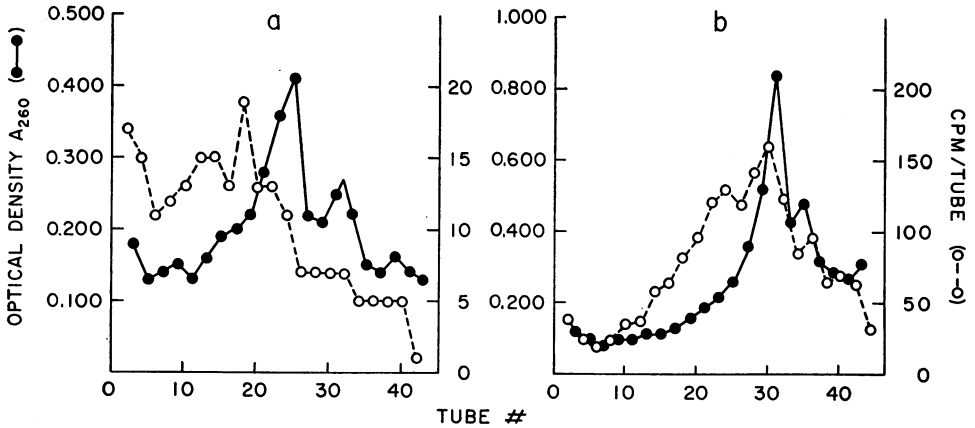

FIGURE 3 Sedimentation patterns of labeled RNA from PHA lymphocytes incubated with uridine-^{3}H (10 μc/ml) for 1 (*a*) and 4 (*b*) hr. Uridine-^{3}H was added to the cultures after 6 hr of preincubation with PHA. Gradients were centrifuged for 16 hr at 17,000 rpm in two different centrifuges.

added to the cultures 12 hr before the addition of uridine-^{3}H. The differently labeled RNAs were then run simultaneously on the same sucrose gradient. As shown in Fig. 4a the percentage of radioactivity sedimenting in the 30–60S region of the gradient after a 1 hr incubation with the isotope was essentially the same (70–75%) in unstimulated and PHA-treated cultures. The addition of actinomycin D, 2.5 μg/ml (Merck Sharp & Dohme, West Point, Pa.), after this 1 hr exposure to the isotope, followed by 3 additional hr of incubation, revealed a striking difference between the two cultures as shown in Fig. 4b. In the PHA-stimulated cells approximately 65% of the remaining radioactivity had become associated with the ribosomal and transfer RNA, whereas most of the radioactivity in the unstimulated lymphocytes still sedimented in the 30–60S region of the gradient. This difference was also observed between RNA from stimulated and unstimulated cultures exposed to the isotope for 4 hr without actinomycin D (Fig. 4c).

Further experiments were performed with the actinomycin "chase" in order to study the fate of the rapidly labeled RNA synthesized in the early hours after the addition of PHA. Uridine-^{3}H was added to lymphocyte cultures after 1, 3, 5, and 8 hr of preincubation with PHA. 1 hr of exposure to the label was then followed by a 2 hr "chase" with actinomycin D. The sedimentation

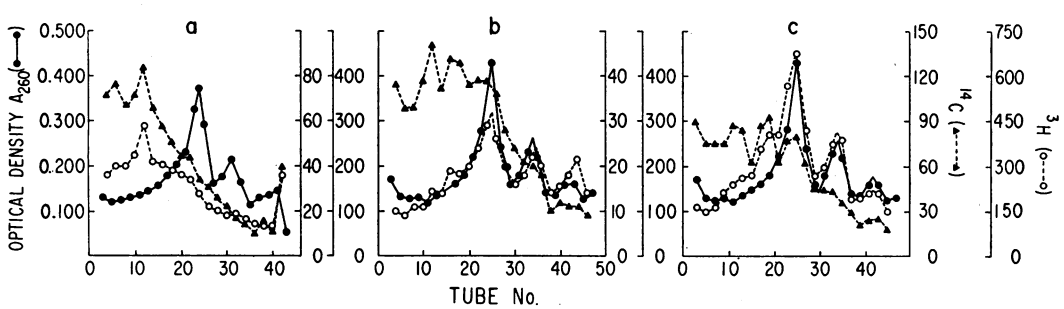

FIGURE 4 Sedimentation patterns of labeled RNA from unstimulated (▲--▲) and stimulated (O--O) lymphocytes before and after actinomycin D "chase." Each pattern was obtained from a single gradient by the simultaneous sedimentataion of RNA extracted from resting lymphocytes labeled with uridine-2-^{14}C. (0.5 μc/ml) and RNA from lymphocytes stimulated for 12 hr with PHA and then labeled with uridine-^{3}H (5 μc/ml). In (*a*) RNA was extracted from cells labeled for 1 hr. In (*b*) 1 hr of labeling was followed by 3 hr of "chase" with actinomycin D (2.5 μc/ml). In (*c*) cells were labeled for 4 hr without actinomycin treatment. Gradients were centrifuged at 17,000 rpm for 16 hr.

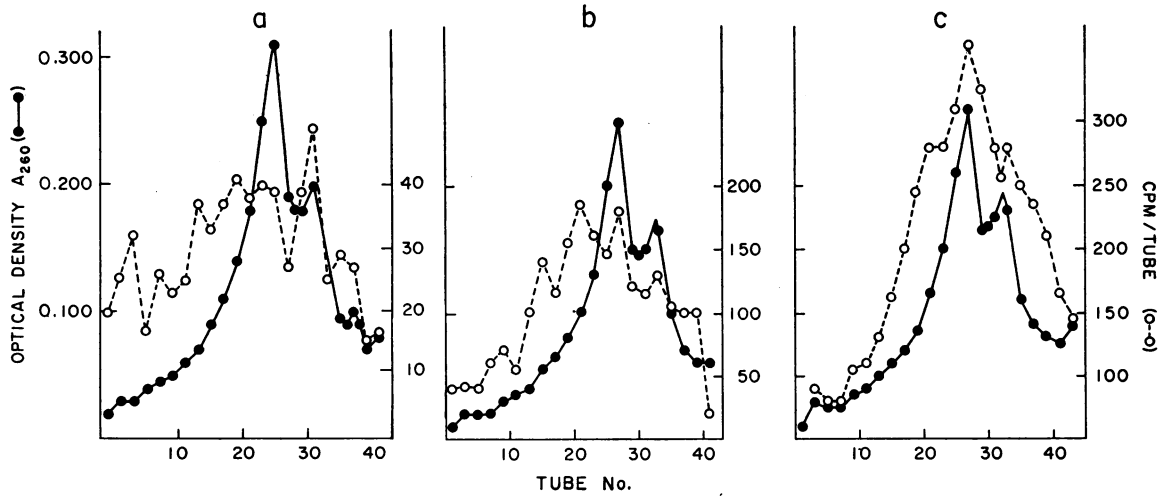

FIGURE 5 Sedimentation patterns of RNA from PHA lymphocytes incubated for 1 hr with uridine-^{3}H (10 μc/ml) and then "chased" for 2 hr with actinomycin D (2.5 μc/ml). The labeled precursor was added, respectively, 1 (*a*) and 5 (*b*) and 8 (*c*) hr after the addition of PHA to the cultures. Gradients were centrifuged at 17,000 rpm for 16 hr.

patterns demonstrated that the rate of transfer of the label from the rapidly sedimenting RNA to "mature" ribosomal RNA was strikingly increased soon after the addition of PHA. Fig. 5 a shows that during a 2 hr actinomycin D "chase" 36% of the radioactivity incorporated in the 2nd hr of exposure to PHA became associated with ribosomal and transfer RNA. A sharp peak of radioactivity at 18S is quite evident at that time. Similar results were obtained from cells labeled during the 4th hr of exposure to PHA. When isotope uptake occurred during the 6th and 9th hr (Figs. 5 b and 5 c) of exposure to PHA, 50 and 60% respectively, of the radioactivity became associated with 28 and 18S ribosomal and 4S RNA during the 2 hr of actinomycin "chase." A distinct labeling of the 28S ribosomal peak is clearly evident in the pattern obtained from cells labeled during the 6th hr of treatment with PHA. Since the period of isotopic labeling and exposure to actinomycin D is the same throughout this group of experiments, the increasing percentage of radioactivity sedimenting with the 28 and 18S RNA appears to be a function of the time of exposure to PHA.

Sedimentation analysis of labeled RNA after incubation of lymphocytes with methyl-^{14}C-methionine. Fig. 6 shows the results of the sucrose gradient analysis of RNA extracted from unstimu-

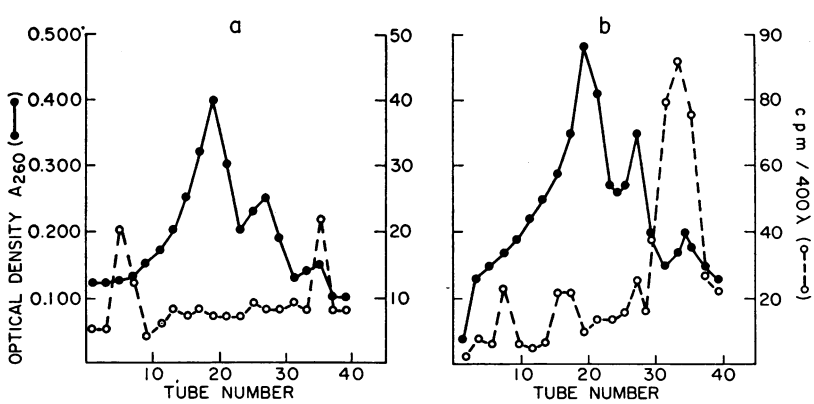

FIGURE 6 Sedimentation pattern of labeled RNA from unstimulated lymphocytes incubated for 30 min (*a*) and 150 min (*b*) with methyl-^{14}C-methionine (0.5 μc/ml). Gradients were centrifuged at 20,000 rpm for 16 hr.

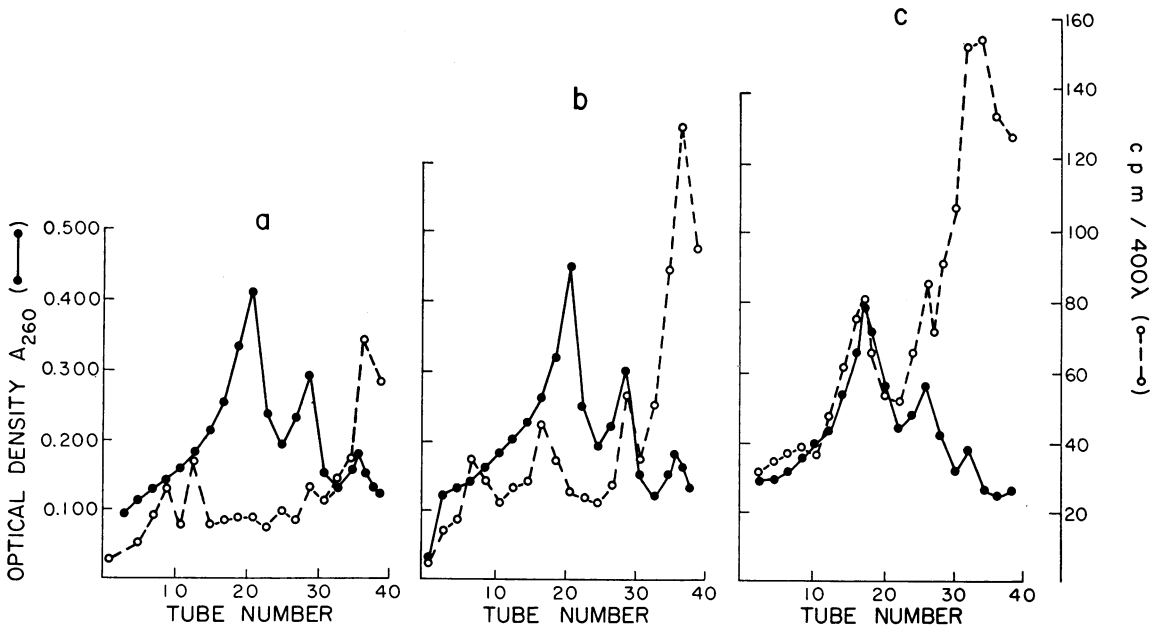

FIGURE 7 Sedimentation patterns of labeled RNA from 12-hr-stimulated lymphocytes incubated respectively for 15 min (*a*), 30 min (*b*), and 60 min (*c*) with methyl-^{14}C-methionine (0.5 μc/ml). Gradients were centrifuged at 20,000 rpm for 16 hr.

lated lymphocytes incubated with methyl-^{14}C-methionine (56.8 mc/mmole) for 30 and 150 min in methionine-free MEM. Two peaks of radioactivity were observed at 45 and 4S after 30 min of exposure to the label (Fig. 6 a). After 150 min two additional radioactive peaks were observed at 35 and 18S, and the degree of labeling in the 4S region was greatly increased (Fig. 6 b). In lymphocytes stimulated with PHA for 12 hr the earliest methyl labeling (except for 4S) was also in the 40–45S region, as revealed by a 15 min incubation (Fig. 7 a). However, the appearance of the methyl label in 35 and 18S RNA was much more rapid, two clear peaks of radioactivity being observed after only 30 min of exposure to the isotope (Fig. 7 b), and after 60 min of incubation the 28S ribosomal RNA peak was distinctly labeled (Fig. 7 c). The level of radioactivity in these experiments was always greatest in the 4S region and presumably represents methylated S-RNA and methioninyl S-RNA. In PHA-stimulated cells pretreated with actinomycin D for 15 min before the addition of methyl-^{14}C-methionine, no significant labeling was observed in RNA species other than 4S RNA. These results represent one of at least three separate experiments, and the findings were quite reproducible. It was thus confirmed that in lymphocytes, as in other cell types, actinomycin D completely prevented the incorporation of radioactive methyl groups into rapidly sedimenting RNA. (It was observed that 30 min of incubation at 22°C with RNase, 50 μg/ml, rendered the radioactivity of the RNA preparation completely acid soluble.)

TABLE I

Hybridization Studies with Normal Lymphocyte RNA

Experiment No.	DNA	Input	RNA-^{3}H	cpm RNA-^{3}H retained on filter (less blank)*	Per cent hybridization
	μg/vial	*μg/vial*	*cpm/vial*		
1	50	18	5500	700	14.7
	50	27	8250	1050	13.2
	50	36	11,000	1220	12.3
	50	54	16,500	1430	8.6
	50	72	22,000	1585	7.2
	50	90	27,500	1640	5.9
2	50	15	2600	600	22.2
3	15	7.5	6050	830	13.8
4	20	10	4500	800	17.7

* The blank vials contained either no DNA or 10 μg of *E. coli* DNA and were always less than 1×10^{-4} of the counts added.

RNA-DNA homology studies. To further

characterize the RNA synthesized in resting and stimulated lymphocyte studies of in vitro RNA-DNA hybridization were performed.

When increasing amounts of pulse-labeled RNA from unstimulated lymphocytes were incubated with 50 μg of DNA, the highest percentage of counts hybridized was observed at an RNA-DNA ratio lower than 0.5. When this ratio was 1.0–1.5 a twofold increase in the amount of labeled RNA reacting increased the number of counts hybridized by only 25% or less, indicating that relative "saturation" of the DNA was being approached. When a similar experiment was performed with pulse-labeled RNA from lymphocytes stimulated with PHA for 24 hr, the percentage of hybridization began to decrease at an RNA-DNA ratio greater than 5.

The maximum percentages of hybridization obtained with different RNA preparations from unstimulated and PHA-treated (24 hr) lymphocytes are shown in Tables I and II respectively. The percentages ranged from approximately 14 to 22% in resting lymphocytes (Table I) and from 2.4 to 4.1% in cells stimulated with PHA (Table II) for 24 hr. The percentage of hybridization in PHA cells is similar to that previously reported for RNA extracted from leucocytes of a patient with infectious mononucleosis (44). When the labeled normal lymphocyte RNA was reacted with DNAs from other sources it was found to hybridize to the extent of 20% of the homologous reaction with mouse DNA, 9% with chicken, and not at all with *E. coli* DNA. These results confirm the specificity of the lymphocyte RNA–lymphocyte DNA hybridization reaction and are compatible with the degree of relatedness among various organisms as demonstrated by DNA-DNA homology studies (45, 46).

Experiments were performed to assay the ability of preparations of unlabeled RNA from resting and PHA-stimulated lymphocytes, KB cells (human epidermoid carcinoma), mouse lymphocytes (L1210 leukemia), chicken oviduct, and *E coli* to "compete" with radioactive lymphocyte RNA in the hybridization reaction with human DNA.

Fig. 8a shows the competitive activity of unlabeled RNA preparations in the reaction of 7.5 μg of radioactive RNA from unstimulated lymphocytes and 5.0 μg of lymphocyte DNA. In each of three separate experiments the unlabeled RNA

TABLE II
Hybridization Studies with PHA-Stimulated Lymphocyte RNA

Experiment No.	DNA	Input	RNA-^{3}H	cpm RNA-^{3}H retained on filter (less blank)*	Per cent hybridization
	μg/vial	*μg/vial*	*cpm/vial*		
1	50	25	32,000	1100	3.4
2	50	15	11,700	480	4.1
3	10	7.5	16,100	590	3.6
4	2	1.5	3075	75	2.4
	2	3	6150	165	2.3
	2	6	12,300	300	2.4
	2	10	20,500	480	2.3
	2	16	32,800	670	2.0

* See Table I for explanation.

from both resting and PHA lymphocytes competed to the extent of 95% of the predicted value for identical RNAs when the ratio of unlabeled to labeled RNA was 10:1. At the same ratio unlabeled RNA from another human cell line (KB) competed to 60% of the predicted value, whereas mouse cell and chicken cell RNAs competed only to the extent of approximately 10 and 18%, respectively. No competition was observed with *E. coli* RNA. These data demonstrate under the experimental conditions used a considerable degree of specificity of the hybridization-competition reaction and suggest that the radioactive complementary RNAs from normal and PHA-stimulated lymphocytes share a high degree of affinity for the same sites on the DNA.

Fig. 8b shows the competitive activity of unlabeled RNA from unstimulated lymphocytes in the hybridization reaction between 15 μg of labeled RNA (24-hr PHA-stimulated lymphocytes) and 3 μg of DNA. Again, it is apparent that the degree of competition at a ratio of 8:1 closely approximates what would be theoretically expected if the labeled and unlabeled RNAs were essentially identical. This result suggests that most of the complementary RNA sequences synthesized in 24 hr PHA lymphocytes were already present in unstimulated cells. Similar results were obtained in each of four experiments with lymphocytes from four donors. The extent of competition by unlabeled KB cell RNA reached a plateau at approximately 65% of the predicted value in this reaction so that part of the complementary RNA

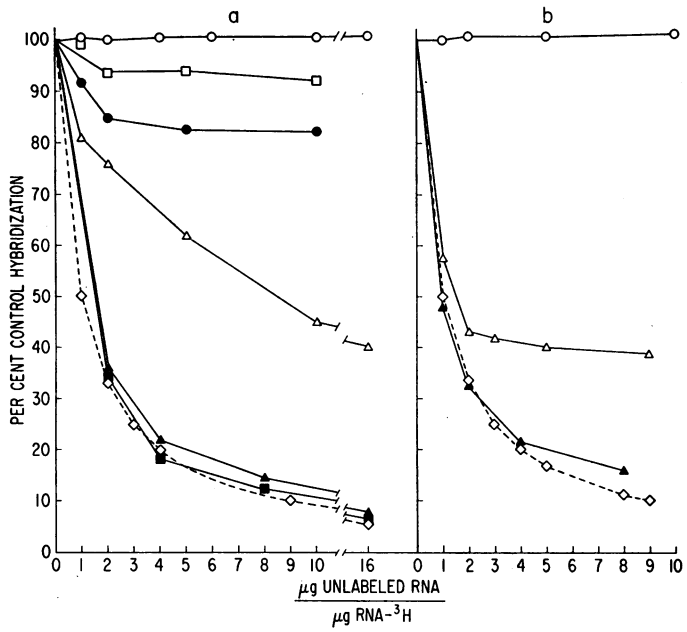

FIGURE 8 (*a*) Competition of unlabeled RNA from unstimulated (▲—▲) and 24-hr PHA-stimulated (■—■) lymphocytes, KB cells (△—△), mouse cells (□—□), chicken cells (●—●), and *E. coli* (○—○) in the hybridization reaction between 7.5 μg of labeled RNA from unstimulated lymphocytes and 5 μg of lymphocyte DNA. The dashed line (◇--◇) represents the theoretical competition curve. (*b*) Competition of unlabeled RNA from unstimulated lymphocytes (▲—▲) and from KB cells (△—△) and and *E. coli* (○—○) in the hybridization reaction between 15 μg of labeled RNA from 24-hr-stimulated lymphocytes and 3 μg of lymphocyte DNA. The dashed line (◇—◇) represents the theoretical competition curve.

sequences in lymphocyte RNA preparations are not present in the KB cell. The *E. coli* RNA was again noncompetitive.

DISCUSSION

Sucrose gradient analysis of the RNA newly synthesized in vitro by unstimulated lymphocytes after 1, 4, 8, and 12 hr of exposure to uridine-^{3}H showed that this RNA consisted primarily of molecular species with a high sedimentation rate. The results also demonstrated that only a small portion of the rapidly sedimenting RNA was converted to 28 and 18S ribosomal RNA in normal lymphocytes. After actinomycin D treatment, part of the newly synthesized RNA was degraded, but no significant amount was cleaved to form "mature" ribosomal RNA. That synthesis of ribosomal RNA did occur in resting lymphocytes is shown by the results of the experiments on methylation of RNA. In fact, after a short time of incubation of normal lymphocytes with methyl-^{14}C-methionine in a methionine-deficient medium a distinct peak of radioactivity appeared in the 45S region, supporting the conclusion that a ribosomal precursor of high molecular weight was synthesized in these cells. After 2.5 hr of incubation two distinct peaks of radioactivity appeared at approximately 35 and 18S, demonstrating that "maturation" of ribosomal RNA took place in circulating lymphocytes, albeit at a considerably slower rate than in PHA cells. There has been some discussion in the literature as to whether the methylation occurs at the 45 or 32S ribosomal RNA precursor (14, 47). It is clear that in the normal lymphocyte the initial incorporation of methyl groups into high molecular weight RNA occurs principally in a discrete RNA fraction whose sedimentation coefficient is approximately 45S, and after longer incubations (2.5 hr) the methyl label was observed in the 32 and 18S RNA fractions.

A seeming discrepancy therefore appears between the results obtained with methyl-^{14}C-methionine and those obtained with uridine-^{3}H, in which no distinct radioactive ribosomal peak was observed even after incubations of 4–12 hr. It seems possible that the cells in which synthesis and maturation of ribosomal RNA occur represent only a very small portion of the total population or, alternatively, one could speculate that most of the cells are synthesizing ribosomal RNA at a very low rate. The high specificity of methyl labeling would allow one to detect the labeling of small amounts of ribosomal RNA, whereas when the lymphocytes are incubated with uridine-^{3}H the small amount of ribosomal radioactivity is overshadowed by the synthesis of larger amounts of nonribosomal RNAs.

In PHA-stimulated lymphocytes the RNA

labeled after 1 hr of incubation with uridine-^{3}H also consisted primarily of molecular species of high sedimentation rate, but after 4 hr of incubation most of the label was associated with ribosomal and transfer RNA. Actinomycin D "chase" experiments with PHA for varying periods of time provided evidence that the newly synthesized, rapidly sedimenting RNA in these cells was converted to 28 and 18S ribosomal RNA. This was already true after just 2 hr of exposure to PHA. Thus, a substantial portion of the rapidly labeled RNA synthesized in PHA lymphocytes may therefore be regarded as a precursor molecule which is rapidly converted to ribosomal RNA. The results of RNA methylation in PHA-stimulated lymphocytes clearly showed that the synthesis of ribosomal RNA proceeded at a much higher rate than in normal lymphocytes, although it followed the same sequential pattern. This result in PHA lymphocytes was quite similar to that described in HeLa cells (14).

The results of the hybridization experiments support the idea that a substantial portion of pulse-labeled (1 hr) RNA synthesized in unstimulated lymphocytes is not a ribosomal precursor. The maximum percentage of hybridization of the rapidly labeled RNA ranged from 14 to 22% in resting lymphocytes, but only from 2.5 to 4.0% in lymphocytes stimulated with PHA for 24 hr. The most likely explanation for the decrease in the efficiency of hybridization after PHA stimulation is that a much larger fraction of the label enters the ribosomal precursor RNA in the stimulated lymphocytes. In fact, if one multiplies the maximum percentage of RNA hybridized by the relative rate of RNA synthesis, one sees that even after 24 hr of PHA stimulation there is only a slight increase in the total labeling of that RNA which was hybridized to DNA.

These changes in RNA metabolism which accompany the cell growth induced by PHA appear quite different from those reported for the regenerating mouse liver (48). Church and McCarthy have shown, in fact, that RNA from regenerating mouse liver has a much higher hybridization efficiency than that from normal liver. Also, in their hybridization-competition studies they were able to adequately distinguish between the RNAs synthesized in the normal liver and regenerating liver after partial hepatectomy. They concluded that a number of species of RNA molecules were synthesized in the regenerating liver which were not present in normal conditions. In our competition studies we were not able to demonstrate, in lymphocytes stimulated with PHA for 24 hr, the presence of RNA sequences different from those present in unstimulated cells. Although the sensitivity of the method is not known and prevents any definitive quantification, these results suggest that the pulse-labeled high molecular weight RNA synthesized in unstimulated lymphocytes includes most of the sequences utilized by the cells in their early development under PHA stimulation. However, with mammalian DNA only a portion of the nucleic acid is reannealed in DNA-DNA homology studies under standard conditions (49). If a similar phenomenon occurred with DNA-RNA hybridization, one might overlook a portion of complementary RNA sequences.

It seems possible to conclude that the majority of circulating small lymphocytes synthesize an RNA of high molecular weight which is not primarily a ribosomal precursor and which is, in part, complementary to human DNA. The macromolecular characteristics of the RNA synthesized in a population of small lymphocytes appear similar to those described in the highly differentiated avian erythroblast (12, 22). The stimulation of these normal lymphocytes by PHA brings a cell population in which very few ribosomes are synthesized to a functional condition in which a large amount of these particles are produced by the cells. This finding is consistent with the morphological observation of a large number of ribosomes in the cytoplasm of the PHA cells (50). It is of interest, in this regard, that several hormones may induce a burst of ribosomal RNA synthesis in their target organs (51). Our results point out that the increase in ribosomal RNA synthesis appears indeed as one of the earliest and most relevant effects of PHA on the RNA metabolism of small lymphocytes. One might speculate that this increase in the synthesis of ribosomes in the PHA cell allows the increased translation of messenger RNA sequences already being transcribed in the normal unstimulated lymphocyte.

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