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Presence of RNA in the Nucleoprotein Complex Spontaneously Released by Human Lymphocytes and Frog Auricles in Culture[1]

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ABSTRACT

Cell systems as different as normal human blood lymphocytes and frog auricles release spontaneously a nucleoprotein complex in their culture medium. This release seems to be an active mechanism that is unrelated to cell death. The presence of RNA in this complex is demonstrated. The amount of extracellular RNA is regulated by the same homeostatic mechanism that has previously been shown to govern DNA release in the same cell systems.

This extracellular RNA is linked by hydrogen bonds to the extracellular DNA and cannot be extracted by a usual phenol procedure, due perhaps to the presence of a glycoprotein. Further purifications by chloroform, sodium perchlorate, and hydroxyapatite are necessary to obtain an RNA molecule that is acid precipitable, RNase and KOH sensitive, and orcinol positive. The extracellular RNA sediments between 2.5 and 4S and is not a transfer RNA. It is more highly methylated than the 28S, 18S, and 4 to 5S cellular RNA. It activates DNA synthesis *in vitro*.

INTRODUCTION

Different cell systems have been found, at least *in vitro*, to release RNA (20) or RNA-containing materials (2, 4, 16, 18).

We have reported extensively a spontaneous release of a DNA-containing complex by biological systems as different as bacteria (31), frog auricles (30-32), and human lymphocytes (5). The release process was unrelated to cell death and was regulated by a homeostatic mechanism. One of the characteristics of this DNA-containing complex lay in its capacity to synthesize DNA in the absence of cells and without the addition of enzymes (3, 6). Several facts indicated that some RNA might also be present in the complex: (*a*) the acellular DNA synthesis was blocked not only by DNase and pronase but also by RNase (3, 6); and (*b*) a polyribonucleotide could be extracted with the DNA from the culture medium of bacteria or frog auricles (2, 4). This molecule, which was difficult to isolate from the complex, had some RNA characteristics. It contained ribose, carried radioactive RNA precursors when the cells had been previously labeled, and annealed specifically to its cellular DNA without having to be denatured. However, this material was not pure RNA, as shown by its resistance to RNase, its incomplete degradation by KOH, and its acid solubility.

The present study will show that not only DNA (3, 5, 6, 30-33) but also a pure RNA can be isolated from the nucleoprotein complex released by cell systems as different as normal human blood lymphocytes and frog auricles. The quantity of RNA in the extracellular medium is regulated by the same homeostatic mechanism that has previously been shown to govern DNA release by the same cell systems (3, 5, 6, 30-33).

MATERIALS AND METHODS

Biological Materials. Human blood lymphocytes were used as a single-cell suspension in TC 199 medium. In experiments with whole organs, frog auricles were used since they can easily survive in Ringer's solution for more than 2 days and have no blood vessels where coagulated blood could remain trapped.

Lymphocyte and Auricle Cultures, Separation of the Supernatants. Human peripheral blood lymphocytes were prepared under sterile conditions by the Ficoll-Isopaque gradient technique (10) with the use of heparinized whole-blood samples from healthy donors. After 2 washings with Hanks' TC medium, the lymphocytes were suspended at a concentration of 10^6 cells/ml in TC 199 buffered with 0.04 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The sources of the chemicals were Pharmacia Fine Chemicals, Uppsala, Sweden, for Ficoll; Difco Laboratories, Detroit, Mich., for Hanks' TC and TC 199; and Calbiochem, La Jolla, Calif., for 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. Sterility was secured by supplementing the medium with penicillin (120 units/ml) (Mycofarm, Delft, Holland) and colimycin 180 units/ml) (Laboratoire Bellon, Neuilly, France). The WBC in such preparations were approximately 98% lymphocytes. Aliquots (35 ml) of the suspension were put into capped plastic tubes (8 tubes/series) and incubated at 37° under air atmosphere for varying times according to the protocol described below. After incubation the lymphocytes were centrifuged a first time at 1000 rpm for 10 min in a Christ Heraeus II KS centrifuge (Martin Christ A. G., Osterhode am Harz, Germany) at 4°. Twenty-five ml of the supernatant were collected by pipetting, leaving 10 ml of medium with the sedimented lymphocytes to prevent cell loss. The former supernatant was then cleared of any remaining cells and cell debris by a second centrifugation at 20,000 rpm for 1 hr in a Sorvall RC 2-B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.). As an ultimate precaution, to

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sediment possible ribosome contamination, a third centrifugation was performed at 50,000 rpm for 2 hr at 4° in a Spinco Model L 75 centrifuge on a Ti 60 rotor (Beckman Instruments Inc., Fullerton, Calif.), and the resultant supernatant was collected and kept frozen until RNA extraction. The lymphocytes put aside after the first centrifugation were resuspended in the remaining 10 ml of supernatant. An aliquot of this suspension was taken for cell counting and for viability tests, as described below. In some experiments, when the lymphocytes were incubated successively several times in fresh medium, 25 ml of TC 199 medium were added each time to the 10 ml of cell suspension for the ensuing incubation period.

Aseptically extracted frog auricles were kept at 20° in aerated Ringer's solution containing penicillin (120 units/ml) and colimycin (180 units/ml). Two hours of incubation were sufficient to remove all blood cells. Two hundred auricles/series were further incubated for varying time periods in 80 ml of fresh Ringer's solution (in the presence of antibiotics). In some experiments the medium was regularly renewed. The auricles were then removed, and the supernatant was submitted to centrifugation, first at 12,000 rpm for 20 min and then at 50,000 rpm for 2 hr as described for the lymphocytes, to remove any cellular debris.

In some control experiments the cells were killed by heating before incubation. Human lymphocytes were incubated at 45° for 12 hr, and the frog auricles were incubated at 50° for 1 hr.

Control of Cell Viability, Cell Counting, and Sterility Tests. Lymphocytes were counted in a hemocytometer at Time 0 and after each incubation. Four separate samples, totaling about 1000 cells for each series, were counted. At the same time the lymphocytes were also examined for their ability to exclude trypan blue. Cells were resuspended at a concentration of 3×10^6 cells/ml, and 2 drops of culture were mixed with 1 drop of 0.2% trypan blue. A total of 400 cells/series were counted in a hemocytometer chamber. The results are expressed as percentage of cells taking up the dye.

In some instances the lymphocytes were tested further at the end of various incubation periods for their capacity to synthesize DNA under a mitogen-induced stimulation. For this purpose lymphocytes treated for the same time periods as in the RNA release experiments were compared with fresh control lymphocytes from the same donor. After these various treatments they were incubated for 72 hr at 37° in TC 199 medium containing 20% homologous serum and PHA[3] (PHA-M, Difco), 0.05 ml of the commercial solution per ml of culture, or Con A (Calbiochem), 50 μg/ml of culture. After 12 hr of labeling with [^{3}H]thymidine (1 μCi/ml), the acid-insoluble radioactivity was determined (24). All experiments were performed on cultures in triplicate.

Cell viability of the frog auricles was controlled by determining the pulsation rate at the beginning and at the end of the experiments. Control auricles were also labeled with [^{3}H]uridine during the last hr of a 24-hr culture. In this case labeled cells counted on autoradiographs showed that 100% of the cells were labeled. Moreover, the pellet of the [illegible] ence of cells or cell fragments. Even if each fragment was counted as a whole cell, they could not account for more than 1×10^{-6} of the total cell population.

The sterility of the supernatants was controlled at the beginning and at the end of incubation by plating aliquots on agar nutrient broth (Difco). Controls without antibiotics were performed to ensure that the results were not altered by these compounds.

Labeling and Radioactivity Measurements. [^{3}H]Uridine (47 Ci/mmol; purity, 98%) (Radiochemical Centre, Amersham, England) was added to the cell cultures at a final concentration of 10 to 20 μCi/ml.

The cells were also incubated with modified Eagle's minimal essential medium containing 4 times the usual concentration of amino acids and vitamins and lacking methionine. To this medium were added 10% AB serum (for the lymphocytes only), the usual amount of antibiotics, sodium formate (2 mg/ml) (to reduce the incorporation of the [^{3}H]methyl group into the purine ring) (14), [^{3}H]methylmethionine (40 μCi/ml; 12 Ci/mmol) (New England Nuclear, Boston, Mass.), and [^{14}C]uridine (0.2 μCi/ml; 450 mCi/mmol) (Radiochemical Centre). After RNA extraction radioactivity was counted in the presence of the suitable phosphor in a Beckman Tri-Carb scintillator.

RNA Extraction. Since the usual phenol procedure for RNA extraction did not allow purification of the polyribonucleotide in the supernatants, 4 kinds of extraction procedures for extracellular RNA were tested and compared: (*a*) acid extraction (27); (*b*) phenol extraction (29); (*c*) chloroform extraction (26); and (*d*) a combination of phenol and chloroform procedures [the material in the supernatant was treated by the phenol procedure (29) up to the DNase (Fluka, Buchs, Switzerland) digestion step and then submitted to the complete chloroform method (26)]. After dialysis the material was loaded into a hydroxyapatite column (9) (DNA grade; Bio-Rad Laboratories, Munich, Germany), which was washed successively with phosphate buffer solutions (equimolar amounts of Na_2HPO_4 and NaH_2PO_4, pH 6.8) as follows. First, 150 ml of a 0.03 M solution were used, and then the RNA was eluted with a 0.2 M solution in 1-ml fractions. For further purification the material containing the RNA as indicated by UV absorption at 260 nm was ultracentrifuged in Cs_2SO_4 (34) for 48 hr at 41,000 rpm in a Spinco L 75 centrifuge. For removal of all the Cs_2SO_4, the fractions containing the RNA, as indicated by UV absorption at 260 nm or by radioactivity, were dialyzed against water for 2 days. Moreover Cs_2SO_4 gradients of released material at different stages of extraction were run. In 1 experiment designed to determine the ratio of released RNA *versus* released DNA, a Cs_2SO_4 gradient was performed without prior DNase treatment or hydroxyapatite elution.

As a control TC 199 medium and Ringer's solution were subjected to the same extraction procedure.

The cellular RNA from the human lymphocytes and the frog auricles was extracted either by the phenol technique (29) or by the combination of the phenol and chloroform procedures described above (*d*). The results presented in the tables concerning cellular RNA were obtained by the same method as that used for extracellular RNA extraction. The yield of cellular RNA obtained with such a drastic

[3] The abbreviations used are: PHA, phytohemagglutinin; Con A, concanavalin A.

method is about 4 times lower in the case of lymphocytes and 30% lower in the case of auricles than that obtained with the classical phenol extraction procedure.

In some experiments in which $[^3H]$methylmethionine was used, extensive deproteinization of both medium and cells was performed before RNA extraction and on the RNA after its extraction. The deproteinization was done as follows. The medium or the cells were digested with pronase (500 μg/ml) (grade B, Calbiochem) for 2 hr at 37° or with protease K (Merck, Darmstadt, Germany) (100 μg/ml) in the presence of 1% sodium dodecyl sulfate for 1 hr at 37° before extraction. After purification the RNA was treated with the same enzymes and reprecipitated in ethanol before counting.

Characterization of RNA. The following studies were performed on the RNA extracted from the supernatants. (*a*) RNA concentration was determined on a Beckman Model DB spectrophotometer by UV absorption at 260 nm and by ribose coloration (11). The absence of protein contamination was controlled (21). (*b*) Enzyme sensitivity was tested by acid precipitation (27) after digestion with the following enzymes: 2 hr at 37° with pancreatic RNase (200 μg/ml) (Sigma Chemical Co., St. Louis, Mo.) previously heated at 100° for 10 min; *Aspergillus oryzae* S1 nuclease (250 units/ml) at 20° for 2 hr as described by Harada and Dahlberg (15); pancreatic DNase I (50 μg/ml) at 20° for 2 hr (Sigma); pronase (200 μg/ml) at 37° for 2 hr (Grade B; Calbiochem), previously submitted to autodigestion for 8 hr at 37°; α-amylase (2 units/ml) from *A. oryzae* at 20° for 3 min at pH 6.9 (Sigma). The absence of contamination of the enzymes was controlled on bacterial RNA and DNA, which were sedimented in linear sucrose gradients with or without enzyme treatment. (*c*) KOH (0.3 M) sensitivity was tested at 37° for 24 hr. (*d*) The molecular weight of the extracellular RNA was estimated by zonal centrifugation on linear gradients in the presence of sedimentation markers (23 and 16S RNA) (Miles Laboratory, Slough, England) according to the method described by McEwen (22). (*e*) RNA density was determined by Cs_2SO_4 ultracentrifugation for 7 hr at 41,000 rpm in a SW 65 rotor on a Spinco L 75 centrifuge (34). (*f*) Solubility in 1 M NaCl was determined (13). (*g*) Primer activity of the extracellular RNA for *in vitro* DNA synthesis in the presence of monkey liver DNA template was tested according to the method of Beljanski *et al.* (7).

RESULTS

Table 1, Part A, shows that with the usual phenol extraction, which yields a pure cellular RNA, the material isolated from the supernatants was RNase insensitive, mostly acid soluble, and only partially degraded by KOH. Enzyme deproteinization before RNase or KOH did not abolish this resistance. However, if the material was submitted to α-amylase digestion, about one-half of the radioactive molecules became acid insoluble, 70% of which were degraded by RNase and KOH.

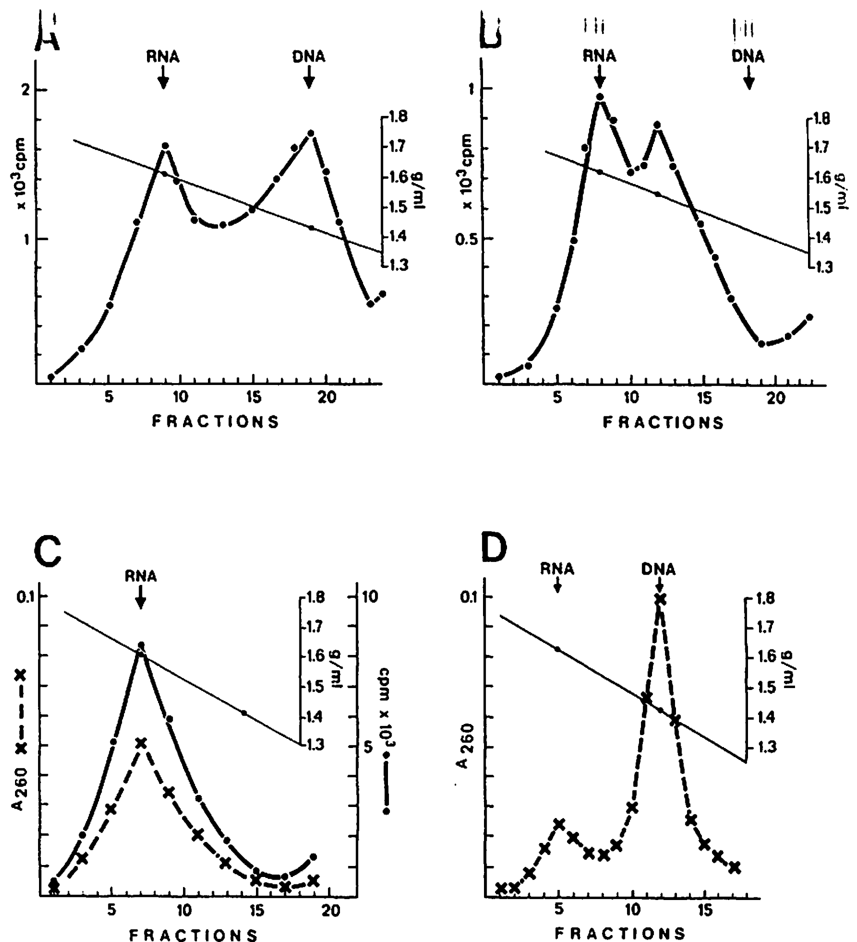
Totally acid-insoluble material sensitive to both RNase and KOH was obtained only when the first phenol extraction was followed by chloroform with a high (1 M) sodium perchlorate concentration (Table 1, Part B). In this case, however, 30% of the radioactivity disappeared after DNase treatment. A Cs_2SO_4 centrifugation of the molecules extracted at this stage yielded 2 radioactive peaks (Chart 1*A*). One peak sedimented at the density of RNA (1.6); the other (60% of the labeled material) sedimented at the level of DNA (1.42). This last peak, although totally degraded by RNase, seemed also partially sensitive to DNase since about 50% of the radioactivity did not precipitate after such a treatment. The same quantity of radioactivity of this second peak was lost after heating at 100° for 10 min followed by rapid cooling on ice. A second Cs_2SO_4 gradient of this peak sedimenting at a density of 1.42 was run after such a

Table 1

State of extracellular RNA After different extraction procedures

	A.	B.	C.
Extraction procedure	Phenol ↓ Ethanol precipitation ↓ DNase ↓ Phenol ↓ Ethanol precipitation	Phenol ↓ Ethanol precipitation ↓ DNase ↓ Chloroform + Sodium perchlorate ↓ Ethanol precipitation	Phenol ↓ Ethanol precipitation ↓ DNase ↓ Chloroform + Sodium perchlorate ↓ Ethanol precipitation ↓ Hydroxyapatite ↓ Cs_2SO_4 gradient
Material extracted	Polyribonucleotide ~50% orcinol positive RNase insensitive <50% KOH sensitive DNase insensitive Acid soluble	Polyribonucleotide ~70% orcinol positive >98% RNase sensitive >98% KOH sensitive ~30% DNase sensitive Acid precipitable	RNA 100% orcinol positive >98% RNase sensitive >98% KOH sensitive DNase insensitive Acid precipitable

Chart 1. Density gradients of labeled and unlabeled nucleic acids at different stages of purification extracted from the culture medium of human lymphocytes. *A*, diagram of a Cs_2SO_4 ultracentrifugation of labeled molecules extracted as in Table 1B (phenol, chloroform, and sodium perchlorate) from the culture medium of lymphocytes labeled with [^{3}H]uridine for 4 hr. *B*, diagram of a second Cs_2SO_4 ultracentrifugation of the peak that sedimented at the level of DNA in *A*. The fractions of the 1.4-density peak that had been obtained after the first run were collected, dialyzed, heated for 10 min at 100°, quickly cooled on ice, and centrifuged again in Cs_2SO_4. *C*, diagram of a Cs_2SO_4 ultracentrifugation of labeled RNA completely purified as in Table 1C (phenol, chloroform, sodium perchlorate, and hydroxyapatite) from the culture medium of lymphocytes labeled with [^{3}H]uridine for 4 hr. *D*, diagram of a Cs_2SO_4 ultracentrifugation of nonlabeled extracellular nucleic acids extracted from the culture medium of lymphocytes after an incubation period of 4 hr. For this experiment the DNase digestion and the hydroxyapatite elution steps were omitted. Centrifugations were run at 41,000 rpm for 72 hr at 20°. After centrifugation 4 drop fractions were collected. Density was determined by measuring the refractive index of the Cs_2SO_4 fractions. A_{260} or radioactivity of the fractions was determined. (Similar results were obtained with frog auricles.)



heating and cooling treatment. After this second centrifugation the majority (70%) of the molecules, which after the first run had a density of 1.42, sedimented at a density of 1.62. The remainder of the radioactivity sedimented between the DNA density region and the RNA density region at about 1.55 (Chart 1*B*).

Elution through an hydroxyapatite column (Table 1, Part C) had to be performed to eliminate the DNA linked to the released RNA. In this case the molecules sedimented after a Cs_2SO_4 gradient as pure RNA in 1 peak (Chart 1*C*).

Nucleic acids recovered after Cs_2SO_4 centrifugation that had not been digested previously by DNase nor passed through an hydroxyapatite column had a RNA/DNA ratio of approximately $^1/_5$ (Chart 1*D*).

The material released from the lymphocytes or from the auricles could be considered, after total purification, as RNA for the following reasons: (*a*) it had a typical UV absorption pattern (260/280 nm ratio = 2.2 and 260/230 nm = 2.5); (*b*) its amount, as determined by UV absorption, was fully confirmed by ribose coloration; (*c*) while more than 98% of it was digested by RNase or hydrolyzed by KOH, it was insensitive to DNase or pronase.

Table 2 (Parts A and B) and Table 3 (Parts A and B) show that the same amount of RNA was recovered in the supernatant whether the incubation lasted for a short or a long time. Similar quantities of extracellular RNA were found after each medium renewal (Table 2, Part B, and Table 3, Part B), whereas when the lymphocytes (Table 2, Part C) or the auricles (Table 3, Part C) were submitted to the same manipulations but resuspended in the old medium there was no significant increase of extracellular RNA.

Chart 2 demonstrates that medium renewal favored the survival of the auricles.

As indicated in Table 4, the supernatant of either a suspension containing only dead lymphocytes or of a culture containing only dead frog auricles did not yield more extracellular RNA after ultracentrifugation than did a culture containing less than 3% dead cells.

Table 5 illustrates the finding that the amount of RNA in the medium increased sharply during the first hr, reaching a plateau after approximately 2 hr.

No detrimental effect of a prior incubation on the ability of lymphocytes to synthesize DNA upon stimulation with PHA or Con A could be observed. Indeed, [^{3}H]thymidine uptake was the same whether the lymphocytes had been incubated for 4 or for 16 hr with or without medium renewals prior to stimulation or had been immediately stimulated.

No RNA was found in the Ringer's solution or in TC 199 medium before incubation with animal or human material.

The molecular weight of the extracellular RNA was small as the released RNA sedimented between 2 and 4S, depending on the extraction procedure (Chart 3).

The RNA released by human and animal cells banded at

Table 2

Amount of RNA extracted from lymphocytes and from their supernatants after various periods of incubation

Lymphocytes were incubated in 150 ml of TC 199 for periods ranging from 2 to 8 hr continuously (A.) or submitted to 4 successive 2-hr incubations renewed with fresh TC 199 (B.), or submitted to 4 successive 2-hr incubations in the same medium (C.)

Time of incubation	Amount of RNA (μg/culture)			Released RNA (%)		Lymphocyte counts (cells × 10^6/ml)		Dead lymphocytes
	Cellular	Extracellular				Before incubation	After incubation	
A. 8 hr	220	8		3.6		1.42	1.40	1.5
B. 2 hr (1st incubation)		9	31.5	3.9	31.6	1.42	1.44	0.8
2 hr (2nd incubation)		7		3			1.40	1.5
2 hr (3rd incubation)		9		3.9			1.39	1.5
2 hr (4th incubation)	231	6.5		2.8			1.37	2.7
C. 4 successive 2-hr incubations in the same medium	255	9.5		3.7		1.42	1.38	2.3

Table 3

Amount of RNA extracted from frog auricles and from their supernatants after various periods of incubation

Auricles (300) were incubated in 100 ml of Ringer's solution for periods ranging from 4 to 24 hr continuously (A.), or submitted to 6 successive 4-hr incubations renewed with fresh Ringer's solution (B.), or submitted to 6 successive 4-hr incubations in the same medium.

Time of incubation	Amount of RNA (μg)			% of released RNA	
	Cellular RNA	Extracellular RNA			
A. 24 hr	1840	8		0.43	
B. 4 hr (1st incubation)		7.5	44.5	0.41	2.33
4 hr (2nd incubation)		6		0.31	
4 hr (3rd incubation)		8		0.42	
4 hr (4th incubation)		7		0.36	
4 hr (5th incubation)		8.5		0.44	
4 hr (6th incubation)	1907	7.5		0.39	
C. 6 successive 4-hr incubations in the same medium	1790	9		0.50	

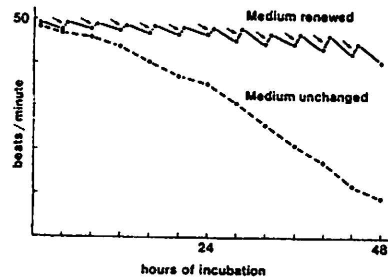


Chart 2. Contraction rhythm of frog auricles in culture with or without medium renewal. Ten auricles/series were incubated in 20 ml of Ringer's solution. In 1 series the medium was renewed at the different times shown by *arrows*. In the other series the medium was not changed. Data plotted on the *ordinate* represent the mean rhythm of 10 auricles.

the same density as the corresponding cellular RNA after Cs_2SO_4 ultracentrifugation.

After treatment with *A. oryzae* S1 nuclease at 20° for 2 hr, while 100% of the control tRNA still precipitated with ethanol, only 25 to 35% of the RNA released from lymphocytes or auricles precipitated after the same treatment. This difference with control tRNA was confirmed by the solubility assay in 1 M NaCl where 100% of the control RNA remained soluble while about 70% of the released RNA precipitated.

Table 6 presents the ratios of [^{3}H]methyl cpm to [^{14}C]uridine cpm in each of the major fractions of cellular RNA separated by sucrose gradients compared to the same labeling ratios in the extracellular RNA. Extracellular RNA has a high ratio of ^{3}H/^{14}C in comparison to the different cellular RNA. Extensive deproteinization performed with pronase or with proteinase K did not modify the ^{3}H/^{14}C ratio of either the cellular or the extracellular RNA.

The extracellular RNA from lymphocytes stimulated *in vitro* DNA synthesis of monkey liver DNA with partially purified *Escherichia coli* DNA polymerase to a level up to 10 times greater than that of the controls without RNA (Chart 4). The frog auricle extracellular RNA had the same effect. However, such positive results could not be repeated at each extraction, and out of 8 medium RNA preparations only 2 had a 10-fold enhancing effect on *in vitro* DNA synthesis, 2 had a smaller effect (2 to 5 times), and 4 had no effect.

DISCUSSION

The results we have presented demonstrate that 2 very different cell systems, namely, nonstimulated human lymphocytes and frog auricles, release, when incubated *in vitro*, a complex containing not only DNA but also RNA. Once completely separated from the different components of the complex, the polyribonucleotide recovered from the medium is a true RNA as evidenced by its sugar coloration, its typical absorption curve in UV, its sensitivity to RNase and KOH, and its insensitivity to other enzymes.

The possibility that the RNA extracted from the complex

of the supernatant could have originated from dying or dead cells is ruled out by several observations that corroborate similar findings on DNA release (3, 5, 6, 30–33). (*a*) The same amount of RNA was found in the medium whether the incubation was of long or short duration, whereas if the phenomenon were due to dying cells one would expect the extracellular RNA concentration to increase with time. (*b*) The amount of RNA in the medium is regulated by a homeostatic system as evidenced by the constant amount of extracellular RNA recovered at each medium renewal. This repeated release of RNA within the complex was shown to be independent of experimental manipulations since there was no significant increase of extracellular RNA when the cells or organs were taken out and put back into the same medium. It should be stressed that after several changes of medium the pulsing activity of the auricles was better maintained than when the medium was not renewed. Thus the treatment allowing longer survival of the auricles in culture also increases the amount of extracellular RNA recovered. (*c*) There is no relation between the cell death rate and the amount of extracellular RNA. When 100% of either the lymphocytes or the auricle cells had been killed by mild heating, there was no significant increase of the extracellular RNA. The RNA recovered in the supernatant of

Table 4

Amount of RNA in the cell-free supernatant of intact or killed lymphocytes or auricles

In Section I lymphocytes (4 × 10^6 cells) were collected and divided in 2 parts. One part (A.) was cultured in 200 ml of tissue culture Medium 199 at 37° for 2 hr under normal conditions. The other part (B.) was killed by heating for 12 hr at 45°. Cell death was checked with trypan blue. The cells were then centrifuged, resuspended in 200 ml of Medium 199, and incubated at 37° for 2 hr. After centrifugation the RNA was extracted from the supernatants, and its amount was determined.

In Section II auricles (900 pieces) were divided in 3 parts. One part (A.) was cultured in 100 ml of Ringer's solution at 20° for 4 hr under normal conditions. The second part (B.), consisting of auricles cut in 4 (but still beating), was cultured as in A. The third part (C.) was killed by heating at 50° for 1 hr. All 3 series of auricles were then incubated in 100 ml of Ringer's solution at 20° for 4 hr and centrifuged. The RNA was extracted from the supernatants, and its amount was determined.

Cells	Treatment	Amount of extracellular RNA (μg/culture)	Dead cells (%)
I. Lymphocytes	A. Normal conditions	8	2.5
	B. Killed by heating	11	100
II. Auricles	A. Normal conditions	7.5	
	B. Cut in 4	4	
	C. Killed by heating	8.5	

Table 5

Amount of RNA extracted from the supernatants of human lymphocytes or of frog auricles after increasing time periods of incubation

Conditions were similar to those described in Table 2 or Table 3, except that the cells were incubated for increasing periods of time. Each incubation time corresponds to a separate culture.

Time of incubation	Amount of released RNA (μg/culture): Human lymphocytes	Frog auricles
10 min	4	2
30 min	6	4.5
1 hr	9	8
2 hr	12.5	9.5
4 hr	9.5	7.5

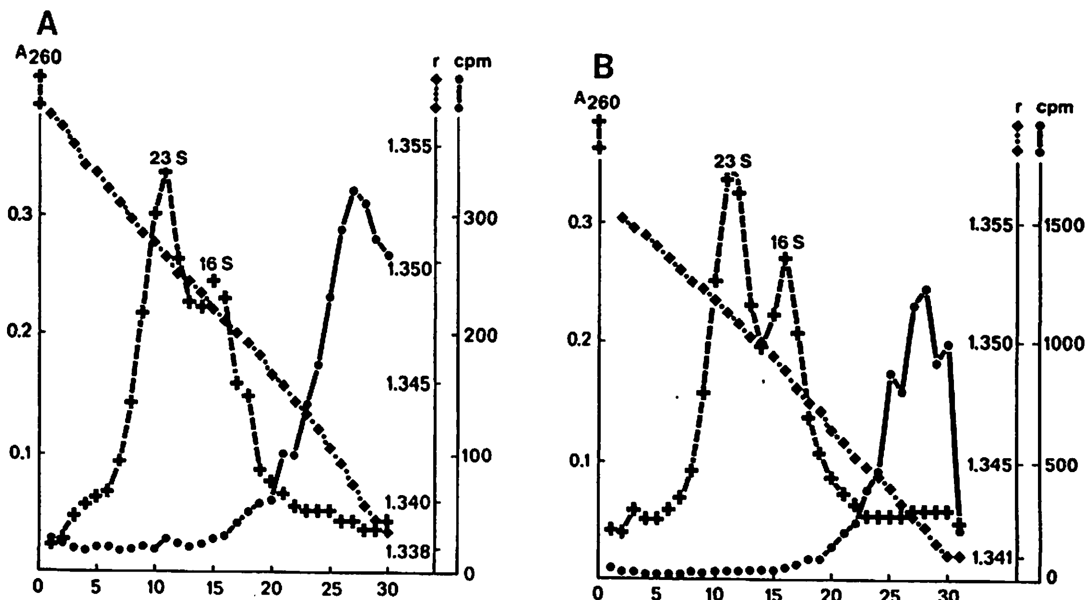


Chart 3. Sucrose gradients of extracellular [^{3}H]RNA extracted from the culture medium of human lymphocytes and frog auricles after 8 hr of labeling with [^{3}H]uridine. The [^{3}H]RNA were centrifuged through a 5 to 20% linear sucrose gradient solution. [^{3}H]RNA (0.2 μg) and 25 μg of *E. coli* rRNA (23S + 16S) as internal standard were charged on each gradient and centrifuged at 19° at 35,000 rpm for 5 hr. Six drops/fraction were collected, and the sucrose density of each fraction was calculated from its refractive index at 20°. The samples were then diluted with 0.2 ml H_2O, and their A_{260} was recorded. The radioactivity in each fraction was counted by liquid scintillation. *A*, [^{3}H]RNA extracted from lymphocyte cultures; *B*, [^{3}H]RNA extracted from frog auricle cultures.

Table 6

Radioactivity of extracellular and cellular RNA of human lymphocytes and of frog auricles labeled simultaneously with [³H]methylmethionine and [¹⁴C]uridine for 16 hr

Cellular RNA (40 μg) or extracellular RNA (10 μg) was centrifuged through a 5 to 20% linear sucrose gradient at 19° for 2 hr at 50,000 rpm. The different fractions of each of the 3 major peaks of cellular RNA and the different fractions of the unique peak of extracellular RNA were pooled, and their radioactivity was counted.

	RNA counted	^3H (cpm)	^{14}C (cpm)	$^3H/^{14}C$ ratio
A. Human lymphocytes	Extracellular	4587	116	39.5
	Cellular 4–5S	5557	1290	4.3
	Cellular 18S	10367	3237	3.2
	Cellular 28S	9550	3292	2.9
B. Frog auricles	Extracellular	2350	104	22.6
	Cellular 4–5S	4723	4217	1.1
	Cellular 18S	4634	4010	1.1
	Cellular 28S	2456	1322	1.9

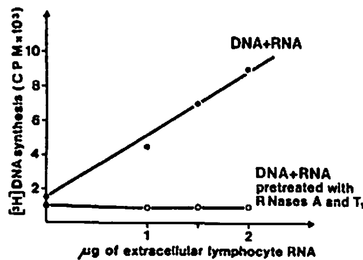


Chart 4. Effect of extracellular lymphocyte RNA on *in vitro* synthesis of monkey liver DNA. The extracellular purified lymphocyte RNA was added to an incubation medium containing monkey liver DNA, *E. coli* DNA polymerase I, and 5 nmol of each deoxytriphosphate precursor. [³H]TTP (50,000 cpm) was added to the medium. *Ordinate*, [³H]DNA synthesis in cpm; *abscissa*, μg of extracellular RNA added to the incubation medium. O, the RNA added to the medium was pretreated with RNases A (50 μg) and T1 (5 μg) for 1 hr at 37°; ●, the RNA was added intact.

the dead cells might have been attached to the membrane and in the process of being expelled when death occurred. However, since the amount of released RNA is equivalent whether the cells have been heat killed or not, it is possible that the regulatory mechanism governing RNA release is not heat sensitive. Moreover, in the case of auricles, the supernatant of wounded organs sliced into 4 pieces does not yield more RNA than does the supernatant of whole auricles. As can be expected from a syncytium, whole or lysed cells are not shed in the medium (it should be remembered that the medium in which the auricles are incubated after excision is discarded since it contains some blood cells and possibly the lysed cells resulting from the ablation of the ventricle). (*d*) Finally, after relatively short incubation periods the cells seem to have maintained good viability properties. The lymphocytes were shown to have kept their functional integrity at the end of the incubation periods as confirmed by their capacity to increase DNA synthesis upon PHA or Con A stimulation just as well as untreated lymphocytes. The viability of the auricle cells was also evidenced by their capacity to incorporate [³H]uridine into their RNA during the last hr of the incubation period. (*e*) In addition, the important difference in methylation between cellular and released RNA is some evidence that the RNA isolated from the complex is a special kind of RNA. Indeed, extracellular RNA appears to be heavily methylated when the methyl/uridine uptake ratio is examined. The finding that cellular and extracellular RNA have the same density after Cs_2SO_4 centrifugation suggests a similar base ratio even if such a gradient might not pick up small differences. Indeed, only a very different ratio could explain the very large methyl/uridine ratio of the released RNA, which seems to be due to an increase in the percentage of methylation not to a decrease in the percentage of uridine.

The extraction techniques that had to be used to obtain a purified RNA suggest that the extracellular RNA is coated differently than the bulk cellular RNA. The different sensitivities of the labeled molecules to enzyme digestion or KOH treatment as observed after the different stages of extraction give some indication of the different components of the complex released by the cells. The acid solubility of the complex or of the partially purified material recovered after the phenol step could indicate the presence of a polysaccharide or of a glycoprotein. This explanation is strengthened by the fact that after an amylase digestion about 50% of the material becomes acid precipitable, 70% of which is RNase sensitive. The presence of a glycoprotein seems more probable than the presence of a free polysaccharide since the polysaccharide can be extracted by chloroform and sodium perchlorate. One could expect the labeled RNA to be sensitive to KOH (although not to RNase) whether or not it is present in a glycoprotein complex. In fact, it might be completely sensitive but still be held together by the complex.

The apparently paradoxical finding of a complete sensitivity to RNase and KOH coupled with a partial sensitivity to DNase of the labeled molecules after a second purification step (chloroform extraction in the presence of sodium perchlorate) can be explained by the Cs_2SO_4 gradients obtained with this material. The first peak sedimenting at a density of 1.6 reacts like pure RNA. The second peak (density, 1.42), which represents about 60% of the radioactivity, although totally sensitive to RNase, is partially sensitive to DNase and to heating. This finding can be explained if one assumes that the peak is composed of very small molecules of labeled RNA attached by hydrogen bonds to larger molecules of unlabeled DNA that have resisted the DNase treatment after the phenol step. Heating separates the 2 nucleic acids and results in the lack of precipitation of the small labeled RNA molecules. The same effect is obtained with DNase destroying the DNA that bridges the small fragments of RNA. This hypothesis is strengthened by the fact that, after heating, most of the precipitable labeled molecules, which first sedimented at the level of DNA, sedimented after a second centrifugation at a density characteristic of RNA. The fact that 23S ribosomal RNA from *E. coli* was not degraded by the DNase we used, as shown by its sedimentation in a linear sucrose gradient, rules out the possibility of an DNase contaminant of the DNase.

After elution through a hydroxyapatite column, the material recovered reacts like totally purified RNA. It thus seems that the RNA is released in a complex with the DNA and that the molecules are also coated with a glycoprotein. The fact that the RNA release is controlled by the same homeostatic mechanism as is the DNA release (6, 31, 32) also argues in favor of an association of the 2 nucleic acids. A heterodu-

plex RNA-DNA has been found within the nucleic acid protein complex released by the water mould *Allomyces arbuscula* (18).

This RNA is single stranded as shown by its elution characteristics on hydroxyapatite. It is of low molecular weight (between 2.5 and 4S). This low molecular weight is probably not due to degradation of the extracellular RNA in the medium by RNase since even after a phenol extraction the molecule is still RNase insensitive. However, it cannot be excluded that the drastic extraction procedures needed to obtain a purified RNA could result in the breaking of the molecule, which might explain the variations we observe between 2 and 4S. This variation in the final molecular weight of the extracted RNA might well be the reason for the absence of its primer activity on *in vitro* DNA synthesis observed in some of our samples. Indeed, if only one 3'-OH terminal group were deleted, all primer activity of the extracellular RNA would be lost. Bulk cellular RNA, of the same size and in the same conditions, does not present this enhancing effect on DNA synthesis. This property is of interest if we remember that in previous studies (3, 6) the activity of the DNA polymerase that was found to accompany, the released DNA was partly RNase sensitive.

The possibility that the released RNA could originate from bacteria or *Mycoplasma* is ruled out by the following arguments: (*a*) culture media were shown to be sterile on agar plating; (*b*) the culture media were centrifuged at 50,000 rpm for 2 hr before extraction, a speed at which such contaminants would sediment.

The extracellular RNA does not seem to be related to any known animal RNA virus. Indeed, no 70S RNA was detected in the medium even if the 50,000-rpm centrifugation was omitted. Moreover, the low-molecular-weight RNA is probably not a viral 4S RNA since it is not a tRNA, as is most 4S viral RNA (25).

The low molecular weight of the extracellular RNA indicates that it is not related to the macromolecular RNA that is transferred from cell to cell in 3T3 cells in culture (19). For the same reasons it is probably also different from "immune" or "informational" RNA (8, 17), which is supposed to migrate from macrophages to lymphocytes (1) or from neoplastic to normal cells (12, 16). On the other hand, there is a similarity between the RNA excreted in the medium by normal and transformed 3T3 fibroblasts described by Kolodny *et al.* (20) and the extracellular RNA studied here. Both have a low molecular weight, both are resistant to RNase before extraction, and both are highly methylated. However, a seemingly purified and acid-precipitable RNA was obtained by Kolodny from the culture medium with the classical phenol extraction. If these 2 RNA's were similar, one should then assume that at least in 3T3 cells, as cultured by this author, some RNA is detached from the DNA-RNA protein complex and is thus easier to isolate. Preliminary experiments performed in our laboratory with cultures of mouse embryo fibroblasts or established HeLa cells did not yield any easily extractable RNA in the supernatant but did yield the same complexed polyribonucleotide as is released by human lymphocytes and frog auricles.

The origin of the extracellular RNA is still unknown. Extensively methylated low-molecular-weight RNA has been reported in cell nuclei (35), and whether some of the cell membrane-attached RNA (28) is not in fact nRNA that has migrated to the cell membrane before being released should be investigated. It should be remembered that membrane-attached DNA migrates from the nuclei to the cell membrane (23).

Whether extracellular RNA is involved in intercellular transfer of specific information or has only an unspecific stimulating function cannot be answered at this stage. The presence in the released complex of an RNA that has a stimulating effect on DNA synthesis *in vitro* seems to be a general phenomenon. The fact that cells of a neuromuscular tissue, fibroblasts, and even established neoplastic cell lines release the same type of RNA as do lymphocytes shows that the phenomenon is not limited to cells taking part in immune response.

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