Purification of RNA from Animal Cells Using Diethyl-Pyrocarbonate[1]

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Summary. Extraction of RNA from animal cells by a method using diethyl-pyrocarbonate yielded 50–60% of the total RNA. RNA purified by a hot phenol-SDS method from adenovirus 2 infected cells showed about 9% homology with adenovirus DNA, and RNA purified by diethyl-pyrocarbonate-SDS showed over 7% hybridization. Profiles of RNA prepared by both methods were identical when studied by polyacrylamide gel electrophoresis.

A simple method for extraction and purification of RNA was sought that would be easy to handle and provide samples from animal cells satisfactory for hybridization studies and size analysis. A method using diethyl-pyrocarbonate (DEPC) for extraction of RNA from plant tissues and bacteria cells was reported[3,4]. In this communication, we report the use of a modification of the DEPC method for animal cell RNA or their virus RNA extraction and compare it with different phenol extraction methods.

Materials and methods. Human KB cells were grown in suspension in MEM supplemented with 5% horse serum[5]. Cells were kept at concentration of $2–3 \times 10^5$/ml. Cynomolgus monkey kidney cells (YCB-1) were grown in roller bottles with 5% fetal calf serum.[6] Rat embryo cells (78A1) transformed by mouse sarcoma-leukemia viruses were grown on monolayer bottles in MEM containing 7.5% fetal calf serum.

Virus infection. KB cell cultures were infected with 50–100 plaque-forming units of adenovirus 2 per cell[5].

DNA. Adenovirus DNA was isolated from purified virus particles as described by GREEN and PINA[7].

RNA. Cell RNA was labeled with ^{3}H-uridine (New England Nuclear Co.) (2–5 μCi/ml, 20 Ci/mmol) for 6 h. KB cells infected with adenovirus 2 were labeled with ^{3}H-uridine (10 μCi/ml) from 2–18 h after infection. RNA was extracted from cell pellets by the following methods: 1. hot phenol-sodium dodecyl sulfate (SDS) method[8]; 2. phenol extraction for cytoplasmic RNA at room temperature after nuclei and mitochondria were removed at 27,000 *g* for 25 min[9]; 3. DEPC-SDS method: The method of SUMMER[4] was modified, the cell pellet was diluted with 15–100 volumes of lysis buffer [0.01 *M Tris*, pH 8.1, $0.1 \times$SSC, 1.5% SDS and 3% diethylpyrocarbonate (DEPC, Sigma Co.)]. The cell suspension was incubated at 37 °C for 5 min and chilled for 10–15 min. One-half volume of saturated NaCl solution was then added and mixed gently. It was then centrifuged at 10,000 rpm for 10 min in a Sorvall centrifuge. The supernatant was pipetted carefully from bottom layer. $2^1/_2$ volumes of 95% ethanol was added to the supernatant and kept at −20 °C overnight. The DNA thread, if it was visible, was removed before centrifugation at 15,000 rpm for 20 min. Precipitated RNA was dissolved in $0.1 \times$SSC.

DNA-RNA hybridization. Denaturation, immobilization of DNA and DNA-RNA hybridization procedures used were as described by FUJINAGA and GREEN[10].

Polyacrylamide gel electrophoresis. Polyacrylamide gels were prepared according to methods described by LOENING[11], BISHOP et al.[12], and WEINBERG et al.[13] containing 2.8% acrylamide, 40 m*M Tris* acetate, pH 7.2, 30 m*M* Na-acetate, 1 m*M* EDTA, 0.08% N, N, N′,N′-tetra methylethylenediamine, 0.08% ammonium persulfate and 10% glycerol. In some experiments agarose was

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Table I. Recovery of RNA from cells by different methods

Sample	Method of extraction	Recovery (%)
KB	Hot phenol SDS	89
	DEPC-SDS	50
Monkey kidney cells (YCB-1)	Hot phenol SDS	88
	DEPC-SDS	60
MSV transformed rat embryo cells (78A1)	Phenol-cytoplasmic RNA	71
	DEPC-SDS (1:30)[a]	55
	DEPC-SDS (1:50)	61
	DEPC-SDS (1:100)	68
Normal rat embryo cells	Phenol-cytoplasmic RNA	65
	DEPC-SDS (1:50)	49
	DEPC-SDS (1:100)	60

[a] Dilution with lysis buffer.

Table II. Hybridization of Ad 2 RNA with homologous viral DNA

RNA		DNA		Radioactivity bond	
Extraction	Input cpm	Source	µg/filter	cpm[a]	%
Phenol-SDS	353700	Ad 2	3	31500	8.9
		none	0	29	0.008
DEPC-SDS	65370	Ad 2	3	4540	7.1
		none	0	27	0.04

[a] Average of duplicate reactions after RNase treatment.

added to a final concentration of 0.5%. Electrophoresis buffer contained 40 m*M* *Tris* acetate, pH 7.2, 30 m*M* Na-acetate, 1 m*M* EDTA, 0.2% SDS, and 10% glycerol. Electrophoresis was carried out at 5 mA per tube for about 3 h. The gel was sliced and solubilized by NH_4OH (gels which contained acrylamide-ethylene diacrylate 15:1) or H_2O_2 (gels which contained acrylamide-*bis*-acrylamide 19:1). In some experiments when cold RNA samples were used, scanning of gels at 260 nm with the Gilford 2410 Linear Transport was performed.

Results. Recovery of RNA from animal cells. The following procedures were used for estimation of RNA recovery. The cell pellet was divided into 2 equal portions and suspended in an equal volume of RSB (0.01 *M* *Tris*, pH 7.4, 0.01 *M* NaCl, 0.0015 *M* Mg Cl_2). 20 µl of this cell suspension was taken for orcinol test or trichloroacetic acid (TCA) precipitation to measure the total amount of RNA or its radioactivity. Different amounts of lysis buffer (15–100 volumes) were added to the cell suspension and RNA extracted by DEPC-SDS procedures as described in materials and methods. Samples for hot phenol-SDS purification were diluted with 15 volumes of 0.05 *M* Na-acetate buffer and RNA was extracted at 60 °C. Samples for cytoplasmic RNA extraction were diluted 20 times with RSB.

Recovery of RNA from various cell lines was estimated (Table I). Hot phenol-SDS was used for whole cell RNA extraction and the yield was 88%. Cytoplasmic RNA, extracted 3 times with phenol at room temperature, was recovered from 65% to 71%. The simple DEPC-SDS procedure yielded 50–70% total RNA, depending on the dilution of the cell pellet with buffer at the initial step. Apparently if the cell pellet were suspended in 100 volumes of lysis buffer and RNA purified by the DEPC-SDS method, recovery of RNA could be as high as after extraction from the cytoplasm by phenol. Recovery might be greater with the DEPC method because DEPC has been shown to be a potent RNase inhibitor[14]. When the DEPC-SDS method was used, visible DNA clumps were removed during ethanol precipitation at −20 °C. Part of RNA, associated with DNA, could be lost during this step. This could be the reason for the relatively low recovery.

Hybridization of adenovirus 2 RNA purified by different methods with homologous viral DNA. Cultures of KB cells were infected with adenovirus 2 and harvested 18 h after infection. RNA was labeled from 2–18 h with

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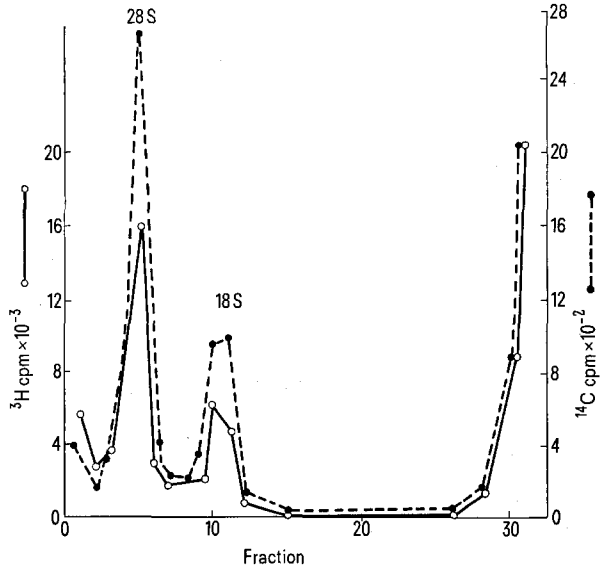

Fig. 1. Polyacrylamide gel electrophoresis of KB cell RNA extracted with phenol-SDS method. KB cells (200 ml, 3×10^5 cells/ml) were labeled with ^{3}H-uridine (10 µCi/ml) or ^{14}C-uridine (2 µCi/ml) for 6 h. ^{3}H-uridine or ^{14}C-uridine labeled RNA was purified by phenol-SDS method and DEPC-SDS method respectively. 100–150 µg of RNA were apllied to 2.8% polymerized acrylamide-ethylene diacrylate (15:1) gels. Electrophoresis was carried out for 3 h 15 min at 5 mA per tube. The gels were frozen, sliced (2 mm) and each fraction was dissolved in 0.3 ml NH_4OH and counted in Bray's solution.

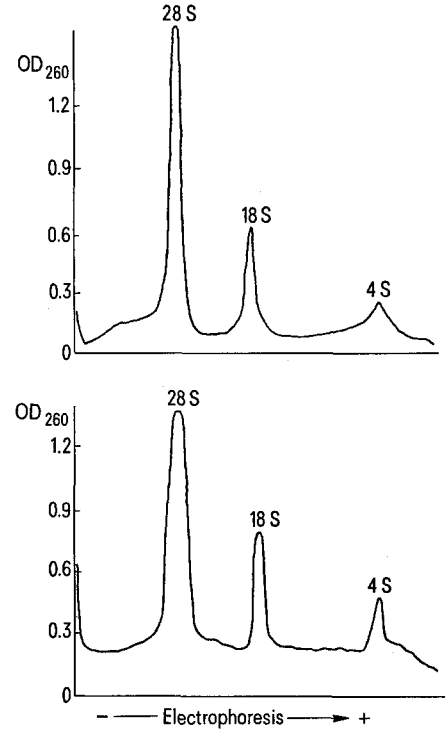

Fig. 2. Polyacrylamide gel electrophoresis of RNA from YCB-1 cells prepared by phenol-SDS and DEPC-SDS method. YCB-1 cells from 2 roller bottles (total 2×10^8 cells) divided equally into 2 tubes and RNA extracted by phenol-SDS and DEPC-SDS method separately. 100-200 µg of RNA were applied to 2.5% polymerized acrylamide-bisacrylamide (19:1) – 0.5% agarose gels. Electrophoresis was carried out for 3 h at 5 mA per tube. The gels were scanned with Gilford 2410 Linear Transport at OD_{260}. Top panel is phenol-SDS and bottom is DEPC-SDS.

^{3}H-uridine. The cell pellet was divided into 2 portions and RNA was extracted by both methods. Purified RNA was annealed with adenovirus DNA on nitro-cellulose filters (Table II). The RNA sample purified by the hot phenol-SDS method showed about 9% homology with adenovirus DNA and RNA purified by DEPC-SDS method also showed over 7% hybridization. In both cases very low background binding to blank filters was found. This result is in agreement with previously reported hybridization analysis of adenovirus RNA prepared with penol[15]. Therefore, RNA prepared with DEPC method was suitable for DNA-RNA hybridization experiments.

Polyacrylamide gel electrophoresis of RNA. The integrity of cytoplasmic RNA prepared by phenol-SDS or DEPC-SDS from various cell lines was studied by polyacrylamide gel electrophoresis. Figure 1 shows the electrophoresis profiles of ^{14}C-uridine labeled RNA prepared by the phenol-SDS procedure from KB cell cytoplasm and ^{3}H-uridine labeled RNA prepared by the DEPC-SDS method from the same cells. The profiles of RNA extracted by both methods are exactly the same. The ratio of 28 S RNA to 18 S RNA in both samples was also very similar. RNA samples prepared by these 2 methods, from cynomolgus monkey kidney cells (YCB-1) showed the same result (Figure 2). Similar results with mouse cells were also obtained. Thus, it is obvious that the DEPC-SDS method is satisfactory for gel electrophoresis studies to determine the molecular weight of RNA and its size distribution.

Discussion. RNA preparations purified by both DEPC and phenol extraction methods showed the same profile with intact 28 S and 18 S ribosomal RNA. Thus, the DEPC method can provide undegraded RNA molecules for size analysis by polyacrylamide gel electrophoresis.

RNA samples, prepared by both methods, from adenovirus 2 infected KB cells annealed with homologous viral DNA with similar efficiency, indicating the suitability of the method for the extraction of viral RNA from infected cells. This results suggests the possibility of applying the DEPC-SDS method for RNA purification with viral or cellular DNA-RNA hybridization experiments.

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A New Substrate for Cultures of Dissociated Primary Rat Brain

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Summary. Different substrates were used to coat plastic petri dishes for the cultivation of dissociated fetal rat brain cells. Only on surfaces which were coated with a mixture of serum and non-reconstituted collagen, did the majority of the inoculated cells attach singly or as aggregates within 24 h. The attachment of the cells was followed by the outgrowth of cellular processes either from single cells or from aggregates in the same time period. This did not occur on collagen or serum treated or on regular plastic dishes. Under the latter conditions a similar outgrowth was observed only after 3–5 days.

A number of different substrates have been used for the cultivation of dissociated brain cells in culture. The most widely used substrate is collagen isolated from rat tendons, which is reconstituted either by ammonia vapors[3] or by riboflavin[4]. Non-reconstituted collagen has also been described as a substrate for nerve cell cultures[5]. Alternatively, polylysine coated surfaces have been recommended to circumvent possible selectivity during the attachment and cultivation of dissociated brain cells[6]. Other workers have resorted to untreated Falcon plastic dishes for the cultivation of dissociated brain cells from different species[7–9].

Here we report the short-term effect of a two-component substrate on attachment and outgrowth of cellular processes from dissociated fetal rat brain cells.

Material and methods. Cell cultures were prepared by a slightly modified Shapiro and Schrier method[7]. Timed Fisher strain rats (17–19 days pregnant, Iffa Credo, Lyon, France) were anesthetized by exposure to CO_2 for 1 min, the fetuses removed and their brain tissue dissociated mechnically by forcing it through a 210 µm nylon cloth glued to a 10 ml plastic syringe. A single cell suspension was obtained by gravity flow through 130 µm nylon cloth. Coated (see below) or uncoated Falcon tissue culture dishes (35 × 10 mm) were used and inoculated with 1.5 mg/cm^2 wet brain tissue (1 ml of a 15 mg/ml cell suspension).

The culture medium consisted of Dulbecco's modified Eagle medium (Gibco), supplemented with 4.5 g/l glucose, 20% (v/v) fetal calf serum (Gibco), 10 units/ml Na-Penicillin G and 10 µg/ml streptomycin sulfate. Cultures were incubated at 37 °C (95% humidity) in 10% CO_2, 90% air.

Petri dishes were coated in the following way. We added either 2 drops of diluted or undiluted serum or 2 drops of a 1 mg/ml collagen solution (calf skin, A grade, Calbiochem) in 0.017 *N* acetic acid or 2 drops of a mixture of fetal calf serum and collagen (1 mg/ml non-reconstituted) in a 1:3 ratio. Serum dilutions were routinely made with H_2O. The liquid was evenly dispersed on the surface of the dish and dried completely at room temperature in a sterile hood under air current. Coated dishes were equilibrated in the CO_2 incubator with 1 ml of serum supplemented medium before being inoculated with the cell suspension.

Results and discussion. When fetal calf serum (5 to 100%) or reconstituted or non-reconstituted collagen were used separately as substrates, dissociated brain cells did not attach to the coated surface within 24 h (Figures A and B). Most of the cells were floating singly or as cell aggregates of variable size. When serum and collagen (1.0 mg/ml) were mixed (1:3, v/v) and used as

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