Selective Precipitation of Ribonucleic Acid from a Mixture of Total Cellular Nucleic Acids Extracted from Cultured Mammalian Cells

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(Received 14 July 1970)

A simple and reproducible method is described for precipitating RNA selectively from total mammalian-cell nucleic acids extracted by the phenol-sodium dodecyl sulphate procedure at pH 8.0. Under specified conditions bulk RNA is precipitated almost quantitatively whereas bulk DNA remains in solution. Minor components of RNA (detected by pulse-labelling and chromatography on methylated albumin-kieselguhr) and rapidly labelled components of DNA containing single-stranded regions are also precipitated. The usefulness of the method is discussed in the context of isolating separately both RNA and DNA from cultured cells that are difficult to obtain in quantity.

Various methods have been devised for isolating pure RNA and DNA from different organisms. The techniques involved may be classified briefly as follows: (a) enzymic degradation of either RNA or DNA in a mixture extracted directly from the cells (e.g. Kruh, 1967; Marmur, 1961); (b) selective precipitation of DNA from partially purified bacterial or mammalian nucleic acids by the use of propan-2-ol (Marmur, 1961) or diphenolic diphosphates (Kirby, 1961); (c) selective adsorption of DNA by hydroxyapatite (Thomas, Berns & Kelly, 1967); (d) chromatographic fractionation, for example on MAK* (Osawa & Sibatani, 1967). However, these methods either are wasteful in that one component of the nucleic acids is degraded or are not convenient for routine purposes. These considerations are important when it is necessary to obtain separately both RNA and DNA from cells that are difficult to grow in large quantities, for example in RNA-DNA hybridization work with primary explants of embryonic cells growing in tissue culture. The present paper describes a method that may be useful in such circumstances.

EXPERIMENTAL

Ehrlich mouse ascites-tumour cells were obtained (Harrison, 1968) and incubated in a suspension of about 2×10^6 – 10^7 cells/ml in Eagle's minimal-essential medium supplemented with bovine serum (10%, v/v) and lactalbumin hydrolysate (0.25%). L cells were grown as monolayers in the same medium. Embryonic mouse fibroblast cells were obtained from eviscerated and

decapitated 16-day-pregnant mice by standard procedures, and grown in supplemented medium also containing human serum (10%, v/v). L cells and embryonic mouse fibroblast cells were used in the exponential phase of growth as the monolayers were nearing confluence, and were re-fed 6–16 h before treatment.

After incubation with labelled uridine or labelled thymidine, the cells were harvested (by scraping in the case of monolayers) at 0°C and the nucleic acids extracted at 0–4°C as follows (Harrison, 1968). Cells were suspended at about 10^7 cells/ml in buffer A (0.14M-NaCl, 10 µg of polyvinyl sulphate/ml, 0.5mM-EDTA, 0.25% purified bentonite and 0.01M-tris-HCl buffered at pH 8.0), before addition of 20% sodium dodecyl sulphate (B.D.H. Chemicals Ltd., Poole, Dorset, U.K., specially pure) to give a final concentration of 0.5%. After standing for 5 min, an equal volume of freshly distilled phenol was added and the mixture shaken for 15 min at full power on a Griffin shaker, before centrifuging at 40000g for 10 min. The aqueous phase was removed, bentonite added to give a final concentration of 0.25%, and the phenol phase re-extracted with half the initial volumes of buffer A and sodium dodecyl sulphate. The two aqueous phases were mixed, re-extracted with an equal volume of phenol and finally precipitated with 2 vol. of ethanol at 20°C. The precipitate was washed with 1 vol. of ethanol-water (3:1, v/v), dissolved in 1 vol. of polyvinyl sulphate (10 µg/ml), made 2% with respect to potassium acetate, and re-precipitated with 2 vol. of ethanol. This purification step was repeated twice.

The nucleic acids were analysed on a column of MAK essentially as described previously (Harrison, 1968), except that the column was one-fifth of the original size. However, in certain cases the elution with aq. NH₃ was replaced by consecutive stepwise elutions with 10 ml of 0.05M-tris-HCl buffer, pH 8.7, 20 ml of 0.75M-guanidinium thiocyanate and then 20 ml of 6M-guanidinium thiocyanate, each buffered with 0.05M-tris-HCl, pH 8.7. Finally, the column was heated to 90°C and the elution

* Abbreviation: MAK, methylated albumin adsorbed on kieselguhr.

Fig. 1. Effect of ethanol concentration on the precipitation of nucleic acids. All samples were isolated from Ehrlich mouse ascites-tumour cells after a 10 min pulse with $[5\text{-}^3\text{H}]\text{uridine}$ (5 Ci/mmole ; $5\text{--}10\text{ }\mu\text{Ci/ml}$). The solution precipitated without ethanol was found to freeze at -20°C . This was allowed to thaw at 4°C before centrifuging. The corresponding points in (a) and (b) are based on E_{260} or radioactivity measurements on the precipitate and supernatant fractions dissolved in polyvinyl sulphate ($10\text{ }\mu\text{g/ml}$).

with 6M-guanidinium thiocyanate continued (cf. Ellem & Rhode, 1969). Radioactivities of samples of eluate were counted (Gill, 1967) and the radioactivities corrected for quenching by salts. In certain cases samples of nucleic acids were treated with ribonuclease (Landy, Abelson, Goodman & Smith, 1967).

Precipitation procedure. All manipulations were performed in a $1.5\text{ cm} \times 10\text{ cm}$ glass centrifuge tube. The nucleic acids were dissolved in 2 ml of 1 mM-MgCl_2 to give E_{260} about 10 before the addition of 0.4 g of potassium acetate. A small magnetic 'flea' was added and the mixture was stirred gently at room temperature on a magnetic stirrer as ethanol was added dropwise to the vortex. After the required amount of ethanol had been added (say, $x\text{ ml}$), the solution was left at -20°C for 18 h, followed by centrifugation until the supernatant was clear. The supernatant was then removed, leaving the precipitate fraction, ($4.8-x$) ml of ethanol was added to the supernatant and the precipitate formed was collected to give the supernatant fraction.

Effect of precipitation on bulk RNA and DNA. Fig. 1(a) shows, in the case of Ehrlich mouse ascites-tumour cells, how the percentage of the total nucleic acids precipitated varied with increasing ethanol concentration. A large proportion of the nucleic acids was precipitated without the addition of ethanol. However, this proportion increased slightly with increasing ethanol concentration, reaching a steady value of about 68% at ethanol concentrations less than a critical limit of 38% (v/v), above which a slight increase in the ethanol concentration caused precipitation of 90–100% of the total nucleic acids. The existence of this critical limit demands that the addition of ethanol during the precipitation stage be very carefully controlled. Thus for routine work, the ethanol concentration was adjusted to 35% (v/v) by adding 0.85 ml of ethanol to the nucleic acids dissolved in 1 mM-MgCl_2 plus $2\text{ M-potassium acetate}$.

Fig. 2 illustrates typical chromatographic analyses of the precipitate and supernatant fractions from Ehrlich mouse ascites-tumour cells. Analogous chromatographs

Fig. 2. Analysis on MAK of nucleic acids from Ehrlich mouse ascites-tumour cells: (a) precipitate fraction; (b) supernatant fraction, obtained by precipitation with 35% (v/v) ethanol. Cells were labelled as Fig. 1. The radioactivity refers to 0.2 ml/tube . Fractions of volume 1 ml were collected at a flow rate of 6 ml/h . Arrows denote the commencement of elution with 0.3% ammonia. After tube 80 the flow rate was doubled. —, E_{254} ; ---, radioactivity. The notation for q_1 and q_2 follows that of Yoshikawa-Fukada *et al.* (1965). Further details are given in the text.

were obtained with L cells and embryonic mouse fibroblast cells. It was evident that in each case the precipitate and supernatant fractions contained almost entirely RNA and DNA respectively. These results were confirmed by the electrophoresis patterns on polyacrylamide of the precipitate and supernatant fractions (Fig. 3). From this combined evidence it is clear that, under the conditions employed, the precipitation procedure gives a very clean separation of bulk RNA and bulk DNA.

Effect of precipitation on minor components of the nucleic acids. For many purposes it is important that minor components of RNA or DNA are extracted and preserved during purification. Consequently attempts were made to determine whether or not minor components of RNA were precipitated with bulk RNA by the method used. Fig. 1(b) shows that under optimum conditions for precipitation of bulk RNA about 95% of the rapidly labelled RNA was precipitated. In Table 1 the percentages of the total activity associated with the various rapidly labelled components of RNA (see Fig. 2) are compared for the total nucleic acids and precipitate fraction (and also, in Ehrlich mouse ascites-tumour cells, for the supernatant fraction). In all cell types there is little evidence for a selective precipitation of any particular fraction of rapidly labelled RNA. Moreover, for Ehrlich mouse ascites-tumour cells there is direct complementary evidence that no particular component of rapidly labelled RNA remains in the supernatant fraction, i.e. DNA. (With embryonic mouse fibroblast cells and

L cells the amounts of nucleic acids isolated were much smaller and thus the radioactivities of the supernatant fractions were not sufficient to permit accurate determination of the radioactivities of the RNA components.) Therefore it may be concluded that the method described precipitates almost quantitatively both the bulk RNA and rapidly labelled RNA.

Further studies were made to determine whether or not rapidly labelled DNA remained in solution during precipitation, as did bulk DNA. Fig. 4 gives the analyses of the precipitate and supernatant fractions obtained of nucleic acids extracted from embryonic mouse fibroblast cells incubated for 15 min with labelled thymidine. These results, supported by similar analyses of nucleic acids from Ehrlich mouse ascites-tumour cells, show that certain components of rapidly labelled DNA (P_2 , β_1 and β_2) were associated exclusively with the precipitate fraction, whereas component P_1 was distributed between the supernatant and precipitate fractions in the approximate ratio 70:30. This finding indicates that any bulk or rapidly labelled RNA in the precipitate fraction could be contaminated with specific components of newly synthesized DNA.

Fig. 3. Analysis of nucleic acids from L cells on 1.5% acrylamide plus 0.5% agarose gels: —, precipitate fraction; ----, supernatant fraction. Gels were prepared in 0.4 cm $\times$ 10 cm cylinders as described by Dingman & Peacock (1968), stained with Methylene Blue and then scanned with a Chromscan. The RNA and DNA components were identified by their selective removal by pre-incubation with ribonuclease or deoxyribonuclease as described by Landy *et al.* (1967).

DISCUSSION

The method described in this paper has been shown to precipitate RNA from a mixture of nucleic acids extracted from various mammalian cells, leaving bulk DNA in solution (Figs. 2 and 3). The purity of the resulting RNA fraction has been examined by chromatography on MAK and electrophoresis on polyacrylamide. The main components of bulk RNA (rRNA and tRNA) are precipitated almost quantitatively. This result contrasts with that obtained with high salt concentrations (e.g. 2 M-lithium chloride) in the absence of ethanol, when rRNA but not tRNA is precipitated (Barlow, Mathias, Williamson & Gammach,

Table 1. *Effects of precipitation on individual components of rapidly labelled RNA*

The Ehrlich mouse ascites-tumour cells and embryonic mouse fibroblast cells were pulse-labelled for 10–15 min with [3 H]uridine (5–25 Ci/mmol; 5–10 μ Ci/ml). The results for L cells are the average of two experiments: one in which the cells were similarly labelled and the other in which the cells were then subsequently 'chased' in 100-fold excess of uridine for 30 min. The nucleic acids were precipitated with 33–38% (v/v) ethanol. Mean values are quoted $\pm$ s.d.

Cell type	Nucleic acid fraction	No. of expts.	% of radioactivity associated with				
			tRNA	q_1	q_2	$q_1 + q_2$	α -RNA
Ehrlich mouse ascites-tumour cells	Total	4*	3 $\pm$ 1	43 $\pm$ 2.5	26 $\pm$ 1.5	69 $\pm$ 2.5	27 $\pm$ 2
	Precipitate	7	4.5 $\pm$ 0.5	35 $\pm$ 4.5	29 $\pm$ 3	62 $\pm$ 2.5	34 $\pm$ 2.5
Embryonic mouse fibroblast cells	Supernatant	6*	2 $\pm$ 0.5	—	—	74 $\pm$ 2.5	24 $\pm$ 2.5
	Total	2	4.5 $\pm$ 0.5	33 $\pm$ 5	25 $\pm$ 6	58 $\pm$ 1.5	38 $\pm$ 1
L cells	Precipitate	—	7 $\pm$ 3	—	—	46 $\pm$ 6	45 $\pm$ 7
	Total	2	7	35	23	58	35
	Precipitate	—	8	—	—	57	35

* Out of a total of seven experiments on which these measurements were made.

Fig. 4. Analysis on MAK of newly synthesized DNA from embryonic mouse fibroblast cells: (a) precipitate fraction; (b) supernatant fraction. Cells were labelled with [$Me\text{-}^3H$]thymidine (5 Ci/mmol; 10 μ Ci/ml) for 15 min. The radioactivity refers to 0.4 ml/tube. The method of elution with guanidinium thiocyanate was used (see the Experimental section). Other details are as in Fig. 2. —, E_{254} ; ----, radioactivity.

1963). Moreover, the minor components of RNA detected by pulse-labelling behave similarly. Such traces of newly synthesized RNA that remain in solution are not specific types of RNA, as judged by chromatography on MAK (Table 1). Further, the components of newly synthesized RNA precipitated are not limited to those components with specific base-ratios or size: component q_1 is the initial 45S precursor to rRNA (Yoshikawa-Fukada, Fukada & Kawade, 1965) and has a high content of G+C; whereas component q_2 , which sediments at about 52S (Yoshikawa-Fukada *et al.* 1965), and components α_1 or α_2 , which have a modal sedimentation coefficient of about 16S, have low G+C contents (Ellem, 1966).

The situation with regard to the behaviour of newly synthesized components of DNA is more complex. Certain components (P_2 , and β_1 and β_2) are precipitated together with RNA, whereas most (about 70%) of component P_1 remains in solution, as does bulk DNA (Fig. 4). Various lines of evidence (P. R. Harrison, unpublished work; Probst, 1969, 1970; Painter & Schaeffer, 1969; Schandl & Taylor, 1969; Berger & Irvin, 1970) point to the conclusion that component P_2 and the β components of rapidly labelled DNA contain extensive single-stranded regions when measured in low concentrations of salt. However, with the present isolation procedures, rapidly labelled DNA resembles double-stranded DNA by binding to hydroxyapatite in low concentrations of salt (P. R. Harrison, unpublished work). It is not known precisely how the

secondary structure of the DNA or RNA components would alter during the change from low to high concentrations of salt. Therefore it is difficult to interpret the present results meaningfully in terms of differences in the secondary structure of the nucleic acids.

Clearly, the mixing of the P_2 and β components of DNA with RNA during precipitation must be examined critically in relation to the effectiveness of the method in separating RNA and DNA. The slight contamination of one component of nucleic acids by non-specific traces of the other is not a problem, for the RNA or DNA fractions can be purified exhaustively by the use of deoxyribonuclease or ribonuclease respectively. However, the loss of single-stranded components of newly synthesized DNA from the total DNA would be serious if these components had specific base-sequences and functions. Although it cannot be proved rigorously, the latter possibility is unlikely; the single-stranded components of DNA probably represent an intermediate stage in the synthesis of all sequences of the DNA genome. In this case loss of partially completed DNA sequences would be essentially random. For such purposes as RNA-DNA hybridization this would not affect the validity of the results obtained.

The work described in this paper was executed during the joint tenure of a Beit Memorial Fellowship for Medical Research and a Research Fellowship at Trinity Hall, Cambridge. The author thanks Professor J. S. Mitchell for his interest and encouragement and Mrs Margaret Williams and Miss Janice Wright for competent technical assistance.

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