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An Efficient Procedure for the Isolation of Polyribosomes from Tissue Culture

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In this paper an efficient procedure is described for the isolation of polysomes from tissue culture cells. Optimal yields, especially of the heavier classes of polysomes can only be obtained at a rather high KCl concentration in the presence of nonionic detergent. Evidence is provided that the lower polysomal yield after extraction at low salt concentration is not due to breakdown of the polysomes by RNAase.

Furthermore, the yield of polysomes is pH dependent only at low salt concentration, while at high salt concentration no influence of the pH is observed. Refreshing of growth medium several hours before harvesting the cells, increases the ratio of polysomes to 80 S monomers.

In general animal polysomes required for studies on protein biosynthesis have been extracted from homogenates of isolated organs. In recent years there has been a growing tendency to make use of tissue culture cells as starting material. To our knowledge only a few efforts have been made to optimize the isolation procedure for these polysomes. The present paper is an account of a systematic investigation to find optimal conditions for the preparation of polysomes from different types of cultured animal cells.

MATERIALS AND METHODS

Triton X-100 was purchased from BDH Chemicals Ltd., sodium deoxycholate from Merck, nonidet P-40 from Shell Corp. and cycloheximide from Koch-Light Laboratories Ltd.

Tissue Culture Cells

Three cell types were used:

a) HeLa cells were grown in suspension at 36 °C at a concentration of $5 \cdot 10^5$ cells/ml in Eagle's minimal essential medium, supplemented with 5% heat-inactivated horse serum and 0.2% (w/v) methylcellulose.

b) Rauscher virus infected mouse spleen and thymus cells were grown in monolayer in one-liter prescription bottles at 37 °C in TC 199 medium supplemented with 23% calf serum.

c) Epithelial cells from calf lens were grown in monolayer in one-liter prescription bottles at 37 °C in TC 199 medium supplemented with 0.4% lactalbumin hydrolysate and 15% calf serum.

Isolation of Polysomes and Sucrose Gradient Centrifugation

In order to "freeze" the polysomal profile, cycloheximide at a concentration of 50 µg/ml was added to all cultures 20 min before harvesting [1]. Cells from suspension culture were poured into ice cold Hank's medium, sedimented 5 min at $700 \times g$ and resuspended in Hank's medium. This procedure was repeated twice. Cells grown in monolayer which were harvested by scraping were prewashed three times with cold Hank's medium. The scraped cells were collected by sedimentation at $700 \times g$ for 5 min. Monolayer cells, harvested by trypsinization, were prewashed three times with tyrode, prewarmed at 37 °C, containing 3 mM magnesium acetate and incubated at 37 °C for 4 min with 0.08% trypsin and 0.8% EDTA in tyrode. The trypsinised cells were washed twice with tyrode, containing 3 mM magnesium acetate.

About 10^7 washed cells were suspended in 0.3 ml of isolation buffer, then 0.3 ml of detergent solution was added, rapidly mixed and the solution was kept at 0 °C for 5 min under occasional shaking. Nuclei were sedimented at $700 \times g$ for 5 min. An aliquot of 0.5 ml of the supernatant was layered directly on a $20.0-30.7\%$ (w/v) isokinetic sucrose gradient in 0.05 M Tris-HCl pH 8.0, 0.08 M KCl, 0.005 M magnesium acetate [2] and centrifuged at 2 °C in a SB 283 rotor of an IEC centrifuge at 41 000 rev./min ($125 \times 10^5 \times g$ min^{-1}) for 70 min.

RESULTS

Harvesting of the Cells

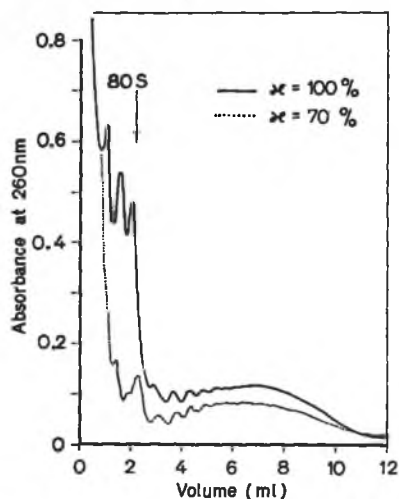Cells were either harvested by scraping them off the glass or by trypsinization. The scraping method was

Fig. 1. *Influence of trypsinizing and scraping on the polysomal yield.* $5 \cdot 10^6$ cells were scraped or trypsinised as described in the Materials and Methods section. The cell-pellet was suspended in 0.3 ml 0.05 M Tris-HCl pH 8.5, 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol and 0.3 ml 1% nonidet P-40 in the same buffer was added. The polyribosomes were isolated and analysed as described in the methods section. The broken line represents polyribosomes obtained by scarping, the solid line the polyribosomes obtained after trypsinization. Direction of sedimentation from left to right. x = relative yield of polysomes

unsatisfactory as many broken cells were detected. Moreover, after labelling the cells with [³H]uridine, about 25% of the radioactive cold trichloroacetic acid precipitable RNA could be detected in the $700 \times g$ supernatant of the cells. This resulted in a lower yield of polysomes as compared with trypsinization, where no rupture of cells occurred (Fig. 1 and Table 1).

Table 1. *Effect of isolation procedure on yield and incorporating ability of polyribosomes from tissue culture*
Incubations were performed for 1 h at 37 °C in 200 µl containing 0.05 M Tris-HCl pH 7.6, 0.08 M KCl, 7 mM magnesium acetate, 0.8 mM ATP, 0.2 mM GTP, 6 mM P-enolpyruvate, 8 µg pyruvate kinase, 5 µM of all amino acids except leucine, 1 µCi DL-[¹⁴C]leucine (specific activity 55.2 mCi/mmol) and 1 mg cell sap of reticulocytes

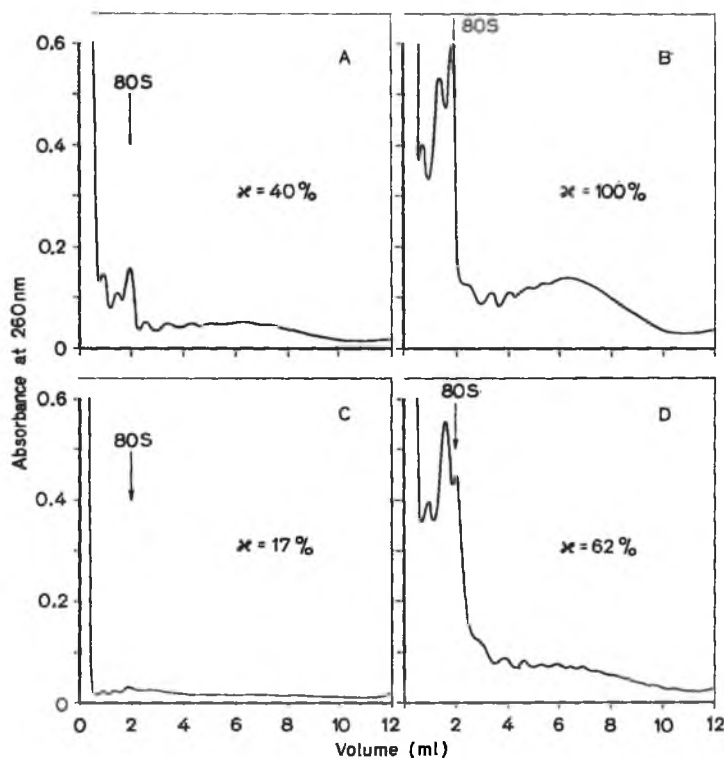| Extraction		Yield rRNA	Incorporation of amino acids into rRNA
Salt	Treatment		
M		µg/10⁷ cells	dis. $\times$ min^{-1} $\times$ µg^{-1}
0.03	trypsin	56	92
0.3	trypsin	87	95
0.03	scraping	25	94
0.3	scraping	70	88

Fig. 2. *Influence of nonidet P-40 concentration and salt concentration on the polysomal yield.* $5 \cdot 10^6$ cells were used. Isolation conditions as described in the methods section. (A) The polyribosomal profile after extraction with 0.05 M Tris-HCl pH 8.5, 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol and 0.01% nonidet P-40. (B) The same buffer as in (A) but with different concentrations of nonidet P-40 in the range 0.05 – 0.5%. (C) Profile after extraction with 0.05 M Tris-HCl pH 8.5, 0.03 M KCl, 0.003 M magnesium acetate, 1 mM dithiothreitol and 0.01% nonidet P-40. (D) The same buffer as in (C) but with 0.05 – 0.8% nonidet P-40. Direction of sedimentation from left to right. x = relative yield of polysomes
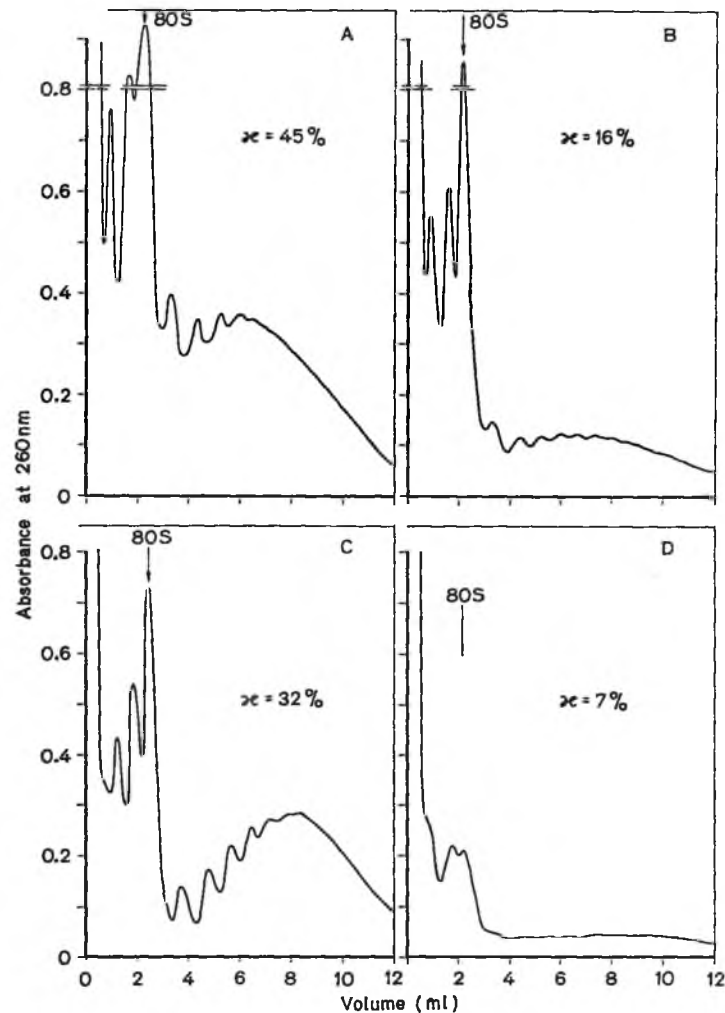
Fig. 3. *Extraction of polyribosomes at low salt concentration and re-extraction of the $700 \times g$ pellet.* (A) $2 \cdot 10^7$ cells were suspended in 0.3 ml 0.05 M Tris-HCl pH 8.5, 0.03 M KCl, 0.003 M magnesium acetate, 1 mM dithiothreitol and 0.3 ml 1% nonidet P-40 in the same buffer was added and polyribosomes were isolated as described. (B) The $700 \times g$ pellet from (A) was re-extracted in the same way as described under (A). (C) The $700 \times g$ pellet from (B) was re-extracted with 0.6 ml 0.05 M Tris-HCl, pH 8.5, 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol without any detergent. (D) The $700 \times g$ pellet from (C) was re-extracted as described under (C) but in the presence of 0.5% nonidet P-40. Direction of sedimentation from left to right. x = relative yield of polysomes

Detergent and Salt Concentration

The influence of the nonionic detergents nonidet P-40 and triton X-100 and of the ionic detergent deoxycholate on the extraction of polysomes from tissue culture cells was examined. When the extraction was carried out in the presence of nonidet P-40 or triton X-100, ranging from 0.05 to 0.8%, the same recovery of ribosomal material was obtained (Fig. 2). However, the yield of polysomes in the presence of detergents was strongly dependent on the ionic strength. For instance when polysomes were isolated in the presence of nonidet P-40 at high salt concentration (0.30 M KCl) consistently higher yields were obtained

as compared with the extraction at low salt concentration (0.03 M KCl) (Fig. 2 and Table 1).

Several authors have suggested that the lower yield may be due to degradation of polysomes [3,4]. This suggestion could be ruled out by the observation that significant amounts of polysomes remained in the $700 \times g$ pellet at low ionic strength. These polysomes could not be released by reextraction of the nuclear pellet at low ionic strength. In contrast at high ionic strength the remaining polysomes could be extracted. Surprisingly the presence of detergent was not necessary for this extraction (Fig. 3).
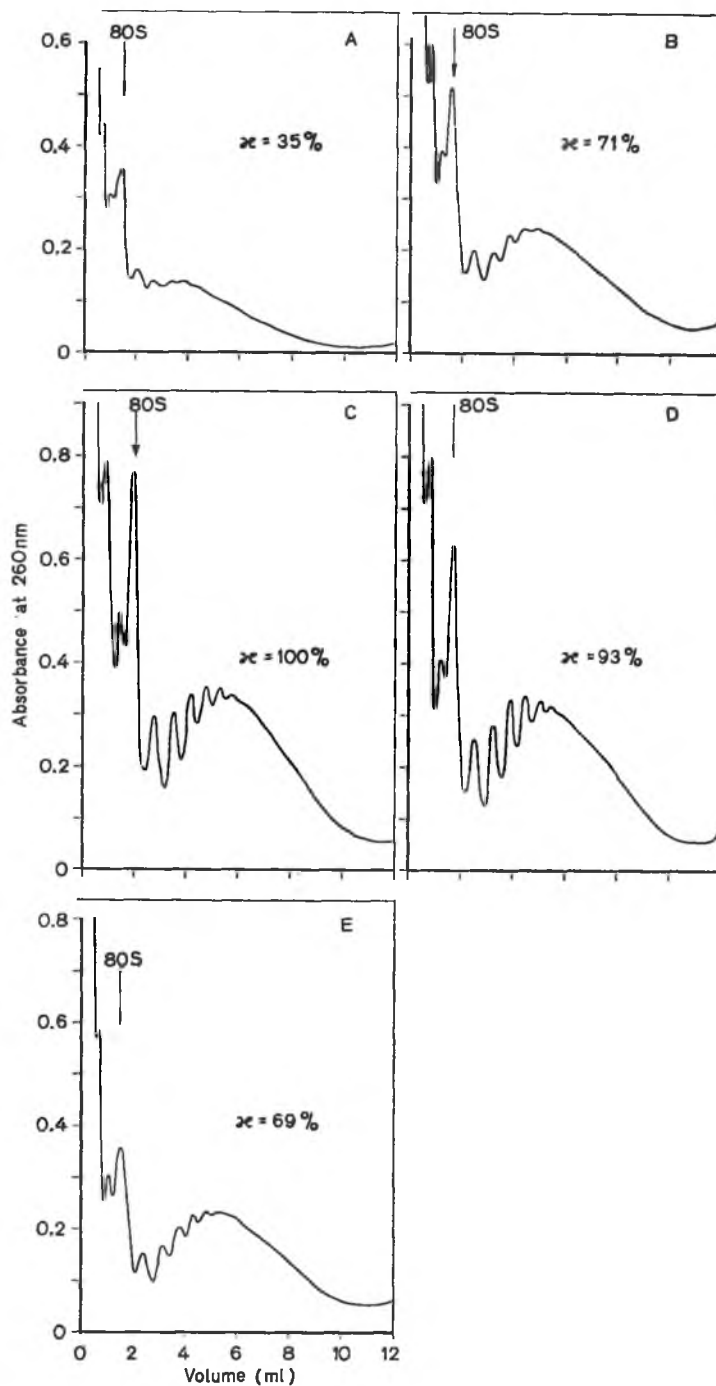
Fig. 4. *Extraction of polyribosomes at different salt concentrations*. 10^7 cells were suspended in various buffers in the presence of 0.5°/₀ nonidet P-40 and polyribosomes were isolated as described. All buffers contained 0.05 M Tris-HCl pH 8.5 and 1 mM dithiothreitol plus (A) 0.01 M KCl and 1.5 mM magnesium acetate, (B) 0.055 M KCl and 4 mM magnesium acetate, (C) 0.15 M KCl and 7 mM magnesium acetate, (D) 0.30 M KCl and 10 mM magnesium acetate and (E) 0.40 M KCl and 12 mM magnesium acetate. In order to obtain a nearly constant free Mg^{2+} concentration a correction was applied for the binding of magnesium ions by chloride [5]. Direction of sedimentation from left to right. $x =$ relative yield of polysomes
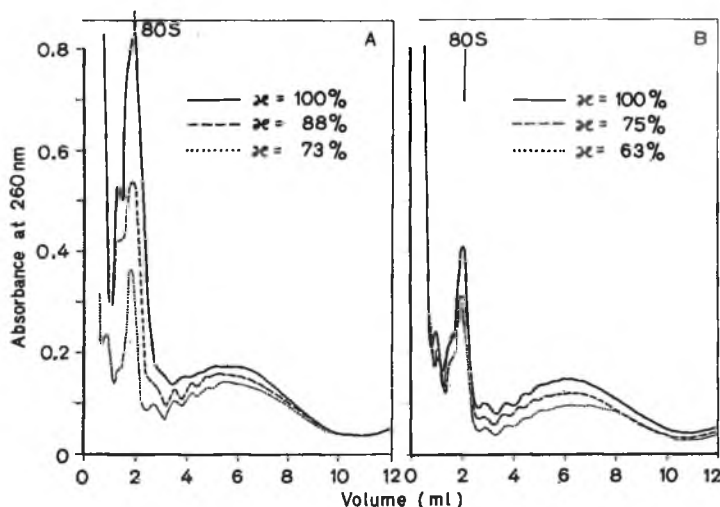
Fig. 5. *Extractions of polyribosomes with deoxycholate as detergent.* $5 \cdot 10^6$ cells were used. Isolation conditions as described in the methods section. (A) The polyribosomal profile after extraction with 0.05 M Tris-HCl pH 8.5, 0.03 M KCl, 0.003 M magnesium acetate, 1 mM dithiothreitol and the following deoxycholate concentrations: ——, $0.5^0/_0$; – – – – – $0.2^0/_0$; ····· $0.1^0/_0$. (B) The polyribosomal profile after extraction with 0.05 M Tris-HCl pH 8.5, 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol and the following deoxycholate concentrations: —— $0.1^0/_0$; – – – – $0.2^0/_0$; ····· $0.5^0/_0$. Direction of sedimentation from left to right. x = relative yield of polysomes
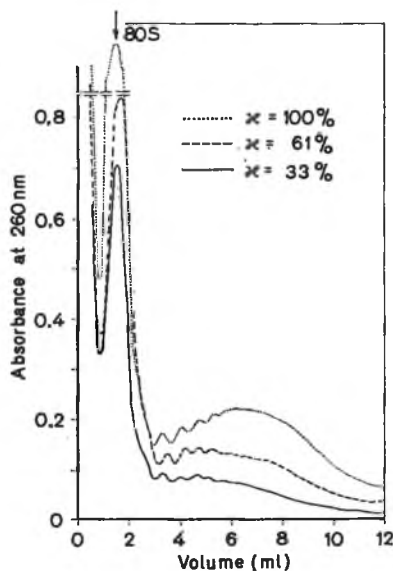

Fig. 6. *Influence of pH on extraction of polyribosomes.* $5 \cdot 10^6$ cells were used. Isolation conditions as described in the methods section. ——, extraction with 0.05 M Tris-HCl pH 7.0, 0.03 M KCl, 0.03 M magnesium acetate, 1 mM dithiothreitol and $0.5^0/_0$ nonidet P-40; – – – –, extraction with 0.05 M Tris-HCl pH 8.5, 0.03 M KCl, 0.003 M magnesium acetate, 1 mM dithiothreitol and $0.5^0/_0$ nonidet P-40; ·····, extraction with 0.05 M Tris-HCl pH 7.0, 0.3 M KCl, 0.01 M magnesium acetate, 1 mM dithiothreitol and $0.5^0/_0$ nonidet P-40. The same curve was obtained at pH 8.5. Direction of sedimentation from left to right. x = relative yield of polysomes

The influence of the salt concentration on the polysomal extraction is shown in more detail in Fig. 4. It can be seen that the optimal concentration is reached at 0.15—0.30 M KCl. Substitution of KCl by NH_4Cl did not influence these results. The effect of the ionic detergent deoxycholate was also studied for the extraction of polysomes from tissue culture cells. At low salt concentration (0.03 M KCl) more polysomes were extracted at higher deoxycholate concentrations (Fig. 5 A). Unfortunately at low salt concentration the nuclei were damaged with deoxycholate concentrations higher than $0.1^0/_0$. At high salt concentration a low deoxycholate concentration was favourable (Fig. 5 B), while in the absence of deoxycholate no polysomes could be extracted. Solubilization of the nuclei occurred at all deoxycholate concentrations tested at high salt concentration. Hence deoxycholate is not satisfactory when it is used as the sole detergent. When deoxycholate was used in combination with nonidet P-40 the nuclei remained intact. With the latter combination a low salt concentration was favourable. For instance with a mixture of $0.5^0/_0$ deoxycholate and $0.5^0/_0$ nonidet P-40 the same amount of polysomes was extracted at 0.03 M KCl as with nonidet P-40 alone at 0.30 M KCl. The combined treatment with deoxycholate and nonidet P-40 at high salt concentration is unfavourable as the recovery of polysomes decreased. The use of a tight-fitting Dounce homogenizer, as described by Penman [6], resulted in significantly lower yields of polysomes
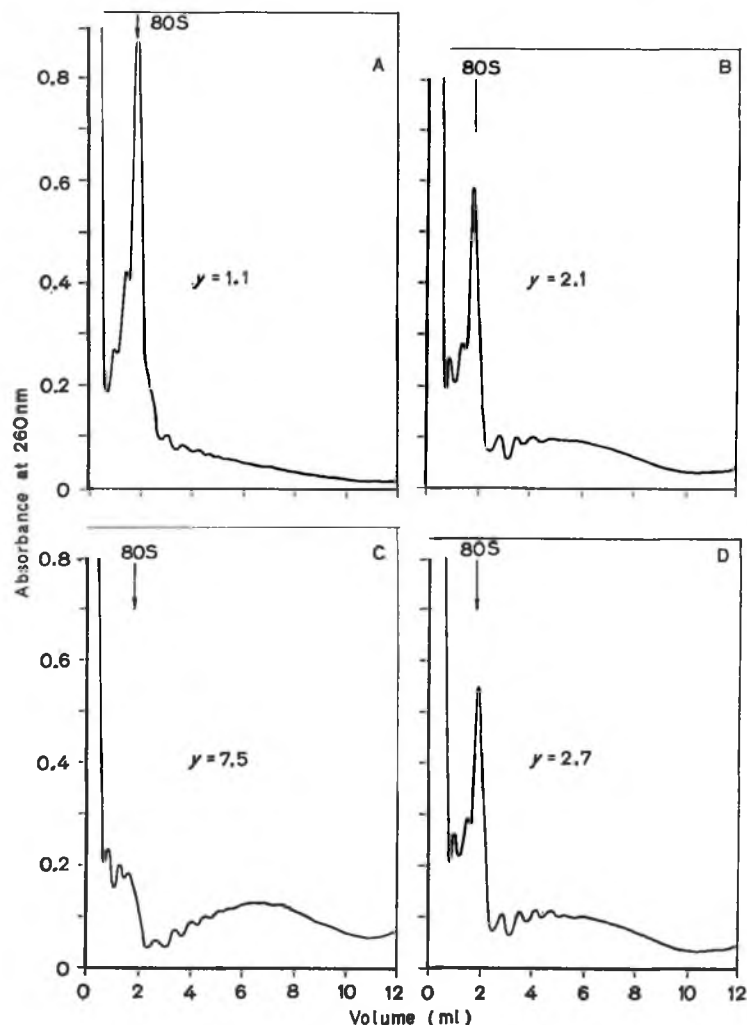
Fig. 7. *Influence of refreshing of the medium on the yield of polysomes from tissue culture cells grown in monolayer.* $3 \cdot 10^6$ cells were used. Isolation conditions were as described in the methods section. Polyribosomes were extracted with 0.05 M Tris-HCl pH 8.5, 0.1 M KCl, 0.2 M NH$_4$Cl, 0.01 M magnesium acetate, 1 mM dithiothreitol and 0.5% nonidet P-40. Curves show polyribosomal profile (A) 24 h, (B) 30 min, (C) between 1 and 6 h and (D) 12 h after refreshing of the medium. $y =$ yield of polysomes/80 S monomer

when compared with the extraction procedure with nonidet P-40 at high salt concentration.

Influence of pH

Extraction of polysomes was performed at pH 7.0, 7.6 and 8.5 in the presence of various salt concentrations. At high salt concentration the amount and size of polysomes extracted were the same at each pH tested. At low salt concentration a significant pH dependence was observed. Most of the material was extracted at high pH values (Fig. 6). However, the total amount extracted was still below the amount extracted at high salt concentration. Once again this decrease in yield at low salt concentration might be due to breakdown by RNAase. This assumption was

verified by examining the influence of the partially purified RNAase inhibitor from rat liver [7,8]. Addition of this inhibitor did not increase the recovery at low salt concentration at either pH value. When the $700 \times g$ pellet, obtained after extraction at pH 7.0 and low salt concentration, was reextracted at high salt concentration the "lost" polysomes were recovered.

Refreshing of Growth Medium

It is well known that protein biosynthesis is strongly influenced by the nutritional conditions of the cell. Polysomal profiles are shifted to the 80 S region as soon as some compounds are exhausted in the growth medium.

Cells harvested 24 h after refreshment of the medium contain only a small amount of polysomes. As can be seen in Fig. 7 even at 30 min after refreshment of the medium a significant shift of 80 S to polysomes occurs. Optimal amounts were obtained 1 to 6 h after refreshing. This time effect is somewhat different from that reported by van Venrooij et al. [9] for Ehrlich ascites tumor cells.

DISCUSSION

For the extraction of polysomes from tissue culture cells two steps are critical: lysis of the cells and the removal of the polysomes from the cell components. In several laboratories a Dounce homogenizer, detergent treatment with hypotonic buffer [6,10—15] and detergent treatment with hypertonic buffer [16,17] is applied.

Cells can be lysed completely, either with buffers of low ionic strength in combination with detergent or with buffers of high ionic strength. However we observed that at low ionic strength the polysomal yield was far less than at high salt concentration. We were able to show that RNAase is not responsible for the low amount of polysomes extracted at low salt concentration. Probably the polysomes are liberated more easily from the cell components at high salt concentration.

For cells grown in monolayer it appears to be disadvantageous to scrape the cells as about $25^0/_0$ of the cells are broken by this method. Trypsinization results in higher yields of polysomes without affecting the amino acid incorporation ability of the polysomes (compare Table 1).

No extraction procedure for polysomes can be considered to be optimal if the final preparation is contaminated with nuclear material. Lysis of [^{3}H]-thymidine labelled cells by nonidet P-40 in the presence of either high or low salt concentration, did not result in nuclear breakage as less than $1^0/_0$ of the radioactivity could be detected in the nuclei-free $700 \times g$ supernatant. In contrast the use of deoxycholate as sole detergent is not convenient for the extraction of polysomes as it causes relatively high nuclear contamination.

It is interesting to notice that extraction at $0.05^0/_0$ nonidet P-40 resulted in the same polysomal yield as at $0.5^0/_0$ and that in this range the amount of polysomes extracted is only dependent on the salt concentration. Whether the nonidet P-40 concentration is critical at higher cell concentrations has not been examined. Refreshment of growth medium at least one hour before harvesting is advantageous as the polysomal profiles are optimal and appear to lack monomers. Apparently cells grown in monolayer respond earlier to nutritional conditions than cells in suspension. Optimal profiles were observed even 1 h after refreshment of the medium of monolayer cells. This deviates from cells in suspension which require several hours to yield optimal polysome formation [9].

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