A Procedure for the Quantitative Recovery of Homogeneous Populations of Undegraded Free and Bound Polysomes from Rat Liver†

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ABSTRACT: A procedure is described for the preparation of free and bound polysomes from whole homogenates of rat liver tissue. Liver is homogenized in a conventional medium containing glutathione; then after a 12-min centrifugation at 131 000*g*, the free polysomes in the supernatant are saved, while the membrane-bound polysomes in the pellet are suspended in a mixture of ribonuclease inhibitors (cell sap, 250 mM KCl, and glutathione), homogenized in the presence of detergent (Triton X-100), centrifuged for 5 min at 1470*g*, decanted, and treated with deoxycholate; the polysomes in the two supernatants are harvested by centrifugation through sucrose gradients containing 250 mM KCl and cell sap. Free and bound polysomes prepared in this manner are undegraded, equally active in cell-free protein synthesis, and virtually free of ribonuclease, membranous material, glycogen, deoxycholate, completed protein, and cross-contamination. The recovery of polysomes is approximately 95% and the distribution between the free and membrane-bound state is 25 and 75%, respectively. The molecular weight profiles after sodium dodecyl sulfate–acrylamide gel electrophoresis of the polypeptides completed and released by free and bound polysomes in vitro are different, indicating that there are quantitative differences in the synthesis of various size polypeptides between the two polysome classes. The differential centrifugation procedure is rapid and reproducible, requires much less ultracentrifugation than the isopycnic technique, and provides a nearly quantitative means of separating free and bound polysomes.

At present the most practical approach for separating free and membrane-bound polysomes is to exploit either the difference in density or size between free polysomes and rough microsomes. Methods based on both these approaches have been developed to separate and quantitate rat liver polysomes (Blobel and Potter, 1967; Howell et al., 1964; Venkatesan and Steele, 1972); however, the isolation of whole, undegraded populations of bound polysomes has not been demonstrated, due largely to the high RNase content of the particulate fraction (Roth, 1967). For this reason most studies have utilized polysomes obtained by isopycnic centrifugation of a postmitochondrial supernatant. This approach is subject to the criticism that the postmitochondrial supernatant contains only a small, possibly nonrepresentative, portion of the bound polysomes (Palade and Siekevitz, 1956; Blobel and Potter, 1967; Venkatesan and Steele, 1972), and that free ribosomes can account for up to 30% of the bound fraction isolated in this way (Blobel and Potter, 1967; Lowe et al., 1970; Bont et al., 1972). In this paper, we present methods for isolating and purifying whole, homogeneous populations of undegraded free and bound polysomes from rat liver.

Experimental Section

Materials

Hepes[1] and bovine serum albumin were obtained from Sigma; sodium deoxycholate, sucrose, salts, and solvents were from Fisher; confectioner's AA sucrose was from C and H; Bacto-casamino acids were from Difco; ribonuclease A and deoxyribonuclease (electrophoretically purified) were from Worthington; creatine phosphate (dipotassium salt) and creatine phosphokinase were from Calbiochem; ATP, GTP, and glutathione were from P-L Biochemicals; ^{14}C- and ^{3}H-labeled amino acids were from Schwarz/Mann and Amersham/Searle; [6-^{14}C]orotic acid was from Schwarz/Mann; [^{14}C]deoxycholate was from ICN; [Me-^{3}H]choline and Aquasol were from New England Nuclear; and NCS was from Amersham/Searle. Amyloglucosidase (Diazyme) was a generous gift from Miles.

Methods

Preparation of Liver Tissue. Livers were obtained from male Sprague-Dawley rats weighing 150 to 200 g (Bio-lab, Minneapolis, Minn.) that had been maintained on the Rockland Mouse/Rat Diet and starved overnight (16–18 h), anesthetized with a circulating air–ether mixture for about 1 min, and perfused via the portal vein with ice-cold 250 mM sucrose–1 mM $MgCl_2$ (40–50 ml) for about 30 s. Livers were pooled in the same medium, and all subsequent operations were performed at 0 to 4 °C. Livers were trimmed, blotted, and weighed. They were forced through a perforated plate (1-mm holes) of a tissue press (Harvard Apparatus Co., Millis, Mass.) to remove fibrous tissue which can decrease the effectiveness of homogenization.

Homogenization. The minced tissue was homogenized with 3 vol (w/v) of polysome buffer (50 mM Hepes, pH 7.4 at 4 °C, 75 mM KCl, 5 mM $MgCl_2$, and 3 mM glutathione) containing 250 mM sucrose in a glass homogenizer with 10 strokes of a motor-driven Teflon pestle (0.006–0.009 in. clearance, size C, A. H. Thomas Co.) rotating at 1750 rpm to obtain a minimum of 95% cell breakage.

Separation of Free and Bound Polysomes. Aliquots of the homogenate (17.5 ml) were centrifuged at 740*g* (2000

† From the Department of Pharmacology, University of Iowa, College of Medicine, Iowa City, Iowa 52242. *Received July 22, 1975.* This work was supported in part by a National Institutes of Health Training Grant (GM-01308) to the Department of Pharmacology, a National Institutes of Health Postdoctoral Fellowship (J.C.R.), and by research grants from the National Institutes of Health (GM-12675 and DA-00710). A preliminary report of this work has appeared (Ramsey and Steele, 1974).

[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; ½-somes, ribosomal subunits.

rpm) for 2 min and then the centrifugal force was increased and maintained for 12 min at 131 000*g* (SW 27.1 rotor, Beckman) as described by Venkatesan and Steele (1972). The supernatant was decanted and saved for the purification of free polysomes. The pellet (particulate fraction) was suspended by homogenization in and adjusted to a total volume of 16 ml with 50% cell sap (a source of natural RNase inhibitor) modified to contain a final concentration of 250 mM KCl (see below). One-ninth volume of 10% (w/w) Triton X-100 was added; then the mixture was homogenized with 3 strokes as above and centrifuged at 1470*g* for 5 min (HB-4 rotor, Sorvall) to sediment nuclei. The resulting supernatant was decanted and mixed with $\frac{1}{9}$ vol of 13% (w/w) sodium deoxycholate to free bound polysomes from membranous material.

Purification of Polysomes. Aliquots of the supernatants (3 ml) containing free and bound polysomes, respectively, were layered over discontinuous gradients comprised of 3 ml each of 1.38 and 2.0 M sucrose (both solutions were prepared in polysome buffer modified to contain 250 mM KCl and cell sap, unless otherwise noted) and centrifuged at 174 000*g* for 20 h (50 Ti or 65 rotor, Beckman) to pellet polysomes. Discontinuous gradients modified to contain cell sap were prepared by the addition of sufficient 50% cell sap to 2.3 M sucrose solutions to give the desired sucrose concentration. After centrifugation the supernatant layers were aspirated and either discarded or saved for analysis. The tube walls were washed with cotton swabs dampened with polysome buffer and dried. The polysomal pellets were suspended in polysome buffer and homogenized by hand with a glass-Teflon homogenizer; for sedimentation analysis the suspension was clarified by centrifugation at 4000*g* for 5 min. Polysomes prepared in this manner have virtually the same size distribution when analyzed either immediately after isolation or after storage (36 h at 0–4 °C, 6 weeks at −20 °C) or after incubation (15 min at 37 °C).

Preparation of Cell Sap. Cell sap for use as a source of natural RNase inhibitor was prepared from fasted rats. A 50% (w/v) homogenate was prepared and centrifuged first at 17 000*g* for 10 min and then the supernatant was centrifuged at 368 000*g* for 95 min or its *g*-min equivalent. The resulting supernatant (upper three-quarters, excluding the lipid layer) appeared to retain full RNase inhibitor activity for at least 2 months at −20 °C and was shown to be ribosome-free when analyzed by the polysome purification procedure described above.

Measurement of Polysome Size Distribution. Aliquots of polysomal suspensions were layered over 15–27.8% (w/w) isokinetic sucrose gradients containing 10 mM Hepes (pH 7.4) at 4 °C, 75 mM KCl, 5 mM $MgCl_2$, and 0.5 mM EDTA and centrifuged at 131 000*g* for 105 min at 2 °C. Sucrose solutions were prepared from commercial sucrose that had been treated with acid-washed charcoal to remove RNase and substances absorbing at 254 nm. After centrifugation the gradients were monitored continuously at 254 nm with an automatic analyzer equipped with a 6-in. recorder (ISCO, Lincoln, Nebr.). To measure the size distribution of polysomes, the recorded absorbance profiles were divided into three segments: (A) area encompassed by subunits to disomes; (B) area encompassed by small polysomes (3 to 8 ribosomes); and (C) area encompassed by large polysomes (>8 ribosomes), and analyzed by planimetry (Model 10 digitizer, Hewlett-Packard). The baseline was virtually flat across the gradient owing to the absence of substances absorbing at 254 nm. The isokinetic gradients used here permit the assignment of fairly accurate sedimentation coefficients (McCarty et al., 1974).

Measurement of Cell-Free Protein Synthesis. The assay medium was prepared essentially as described previously (Venkatesan and Steele, 1972), except that each assay tube contained, in a volume of 2.5 ml: 80 μg of creatine phosphokinase (18 units/mg of protein with creatine as substrate) and 2.5 μCi each of [^{14}C]arginine, [^{14}C]leucine, [^{14}C]lysine, and [^{14}C]valine (312, 312, 310, and 258 mCi/mmol, respectively). The medium was preincubated for 2 min at 30 °C, cell sap (0.2 ml, 6 mg of protein) was added (tenfold excess over rRNA), and incubation was continued for another 2 min, at the end of which polysomal suspension (0.2 ml, 0.6 mg of rRNA, assuming an $E_{1\,cm}^{1\%}$ at 260 nm of 200) was added. For cell sap blanks, polysome suspension buffer (pH 7.3 at 30 °C) was added instead of polysomes. Cell sap prepared from fasted rats was used without Sephadex treatment (Huston et al., 1970). Incorporation of radioactive amino acids into hot trichloroacetic acid insoluble products was determined at various intervals by plating duplicate aliquots of the incubation mixture (100 μl) on filter paper disks and processing them according to the procedure described by Mans and Novelli (1961). Processed disks were digested in 1 ml of NCS at 50 °C for 18 h in scintillation vials and 10 ml of toluene scintillator mixture (6 g of 2,5-diphenyloxazole and 75 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter) was added. Disintegrations per minute (dpm) were determined by the use of a Beckman LS-250 scintillation spectrometer equipped with a dpm controller; ^{14}C efficiency was 67–70% and ^{3}H efficiency was 25–30%.

Comparison of Polypeptides Synthesized in Vivo and in Vitro. Fasted rats were injected intraperitoneally with [^{3}H]leucine (100 mCi/mmol) at a dose of 25 mCi/kg body weight. After 5 min the livers were excised, and the tissue was homogenized and centrifuged as in the preparation of free and bound polysomes. Aliquots of purified polysomal suspensions were incubated for 60 min with [^{14}C]leucine (3.12 μCi/ml; 312 mCi/mmol) as in the cell-free system described above, except that [^{12}C]leucine was omitted. Cell sap was chromatographically treated (Sephadex G10) to remove endogenous amino acids and the cell sap protein to polysomal RNA ratio was increased to 50 in an attempt to promote the release of completed polypeptides (Hicks and Drysdale, 1969). After incubation the mixture was cooled to 0 °C and centrifuged at 200 000*g* for 2 h (SW 56 Ti, Beckman) to pellet ribosomes. Aliquots of the supernatant were precipitated with trichloroacetic acid, washed with acid, dissolved in sodium dodecyl sulfate buffer, and subjected to electrophoresis on 10% acrylamide gels (0.9 × 10 cm with 1-cm 3% stacking gel) at 5 mA per gel according to the procedure of Laemmli (1970). After electrophoresis the gels were stained with coomassie brilliant blue G-250 (Diezel et al., 1972), sliced into 1.8-mm disks, digested in NCS, and acidified with glacial acetic acid, and radioactivity was counted as before.

Preparation of [^{14}C]Orotate-Labeled Free Polysomes and Rough Microsomes. Rats were injected intraperitoneally with [6-^{14}C]orotate (1 mCi/kg, 50 mCi/mmol), fasted for 16 h before being killed, and killed 6 days after injection. A 33% homogenate was prepared and then centrifuged at 10 000*g* for 10 min. Aliquots of the supernatant (3 ml) were centrifuged on discontinuous gradients containing cell sap to separate rough microsomes from free polysomes (Blobel and Potter, 1967). After centrifugation the rough

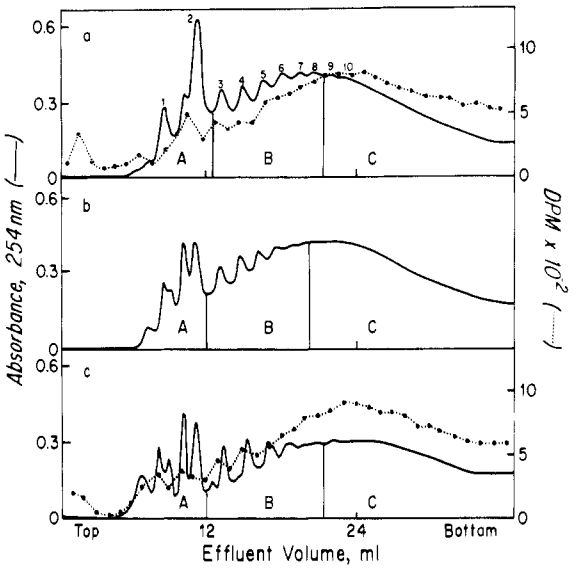

FIGURE 1: Sedimentation profiles of free polysomes from rat liver. Rats were labeled for 5 min with [^{3}H]leucine and a 25% homogenate of liver was prepared and centrifuged as described under Methods. Aliquots of the supernatant were layered over discontinuous sucrose gradients supplemented with either 75 or 250 mM KCl, and with or without cell sap, and centrifuged for 20 h at 174 000g. Pellets were suspended in polysome buffer and aliquots (7.5 A_{260} units) were layered over isokinetic sucrose gradients and centrifuged for 105 min at 131 000g: polysomes purified in the presence of 75 mM KCl without cell sap (a); similar profiles were obtained with polysomes which had been purified in the presence of both 75 mM KCl and cell sap (not shown); polysomes purified in the presence of 250 mM KCl without cell sap (b) and with cell sap (c). Numbers over the peaks correspond to the presumed number of ribosomes in each polysome class. Segments A, B, and C represent areas encompassed by subunits to disomes, small polysomes (3 to 8 ribosomes), and large polysomes (>8 ribosomes), respectively.

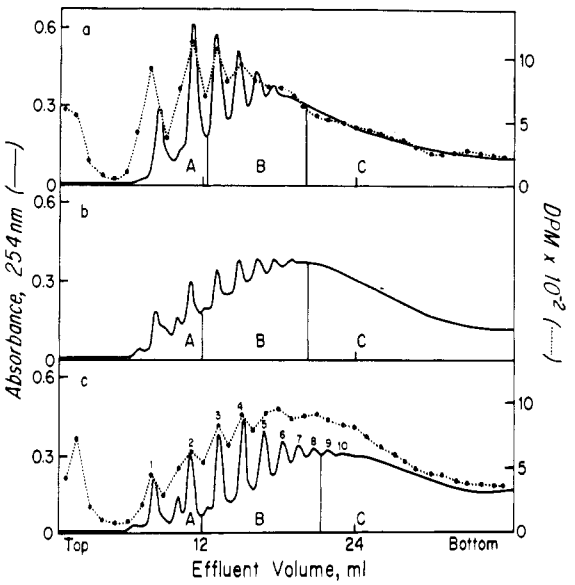

FIGURE 2: Sedimentation profiles of bound polysomes from rat liver. The pellet resulting from the first centrifugation of the homogenate described in the legend to Figure 1 was suspended in cell sap containing either 75 or 250 mM KCl and processed as described under Methods. Aliquots of the supernatant were layered over discontinuous sucrose gradients of the same ionic strength and centrifuged; pellets were suspended in buffer and aliquots (7.5 A_{260} units) were centrifuged on isokinetic gradients: polysomes purified in the presence of 75 mM KCl without cell sap (a); similar profiles were obtained with polysomes which had been purified in the presence of both 75 mM KCl and cell sap (not shown); polysomes purified in the presence of 250 mM KCl without cell sap (b) and with cell sap (c). Other details are as in Figure 1.

microsomes were aspirated from the interface between the 1.38 and 2.0 M sucrose layers, diluted by the addition of polysome buffer containing 5% cell sap to a final concentration of 0.25 M sucrose, and centrifuged at 131 000g for 12 min to pellet microsomes. The pellet was washed three times by homogenization with polysome buffer containing 5% cell sap, centrifuged as above, and then suspended in polysome buffer, as were the pellets containing free polysomes. The ^{14}C-free polysomes and -rough microsomes used in these experiments had a specific activity of 526 000 dpm/mg of RNA.

Measurement of Cross-Contamination between Polysome Fractions. Homogenates were prepared as above, except that before the final strokes were made [^{14}C]orotate-labeled free polysomes or rough microsomes were added to the homogenate and the mixture was fractionated as before. The light (0.25 + 1.38 M) and heavy (2.0 M) sucrose layers of the discontinuous gradients were collected, diluted by the addition of water, and precipitated with 10% trichloroacetic acid. The precipitates were pelleted by centrifugation, digested in NCS for 18 h, and counted as above. The polysomal pellets were solubilized with 0.5% sodium dodecyl sulfate; the latter and aliquots of the acid-soluble supernatants were diluted with Aquasol and counted.

Measurement of Membrane Content of Polysomes. Rats were starved for 16 h and injected intraperitoneally with [Me-^{3}H]choline (2.34 Ci/mmol) at a dose of 50 μCi/100 g body weight (0.5 ml) containing 10 mg of Bacto-casamino acids (Nagley and Hallinan, 1968). After 30 min livers were excised and free and bound polysomes were prepared as above. Aliquots of the homogenate, free polysomes, and bound polysomes were processed on glass fiber disks to remove trichloroacetic acid soluble radioactivity and counted as described by the above authors; some disks were further extracted three times with chloroform–methanol (2:1, v/v) to remove lipids. The specific activity of the homogenate was 6500 dpm/mg of protein.

Chemical Analysis. RNA, DNA, and protein were determined by the method of Fleck and Munro (1962), Burton (1956), and Lowry et al. (1951), respectively. Phospholipids were extracted by the method of Folch et al. (1957) and phosphorus was determined by the method of Chen et al. (1956); the amount of phospholipid was obtained by multiplying the phosphorus value by 25. Glycogen was isolated by KOH–ethanol fractionation of liver homogenates and polysomal pellets and assayed by the amyloglucosidase procedure described by Johnson and Fusaro (1966).

Results

Isolation and Analysis of Free and Bound Polysomes. The method adopted for preparing free and bound polysomes involves centrifugation of liver homogenate to pellet the rough microsomes, homogenization of the pellet with detergent which liberates the bound polysomes and permits the removal of nuclei, and centrifugation of the supernatants to purify the polysomes. The absorbance profiles shown in Figures 1a and 2a confirm the findings of Venkatesan and Steele (1972) that the integrity of free but not of bound polysomes can be preserved by isolating the two fractions in the presence of cell sap (a source of natural RNase inhibitor) and then purifying the polysomes by centrifugation through 75 mM KCl gradients. Thus, either mem-

Table I: Sedimentation Distribution of Polysome Size Classes.[a]

Purification Conditions	Free Polysome Fraction: % Total Absorbance Encompassed by: ½- to 2-somes	3- to 8-somes	>8-somes	Bound Polysome Fraction: % Total Absorbance Encompassed by: ½- to 2-somes	3- to 8-somes	>8-somes
75 mM KCl	20 ± 1	37 ± 1	43 ± 1	19 ± 2	40 ± 1	41 ± 2
75 mM KCl plus cell sap	19 ± 1	38 ± 1	43 ± 0	18 ± 0	44 ± 0[b]	38 ± 0
250 mM KCl	16 ± 1[b,c]	34 ± 1	50 ± 1[b,c]	10 ± 1[b,c]	39 ± 1[c]	51 ± 2[b,c]
250 mM KCl plus cell sap	20 ± 2	30 ± 0[b,c]	50 ± 2[b,c]	12 ± 2[b,c]	38 ± 1[c]	50 ± 1[b,c]

[a] Rat liver polysomes were prepared as described in the legends to Figures 1 and 2 and displayed on isokinetic sucrose gradients, and the resulting profiles from three separate experiments were analyzed by planimetry. The values, mean ± standard error, are not corrected for overlap between segments as indicated in the profiles. [b] Significant difference from 75 mM KCl, $P < 0.05$. [c] Significant difference from 75 mM KCl–cell sap, $P < 0.05$.

Table II: Distribution of RNA in Free and Bound Ribosome Fractions Isolated from 1.0 g Wet Weight of Liver.[a]

Expt	Total RNA (μg/g of Liver)	Free Ribosome Fraction: Heavy Layer μg	Heavy Layer %	Pellet μg	Pellet %	Bound Ribosome Fraction: Heavy Layer μg	Heavy Layer %	Pellet μg	Pellet %	Recovery of Total RNA (%)	Free/Total Ribosomes × 100
1	7930	380	4.8	1180	14.9	620	7.8	3880	48.9	76.4	25.8
2	8850	320	3.6	1060	12.0	950	10.7	3940	44.5	70.8	22.0
3	8200	360	4.4	1280	15.6	810	9.9	3740	45.6	75.5	26.5
			4.3 ± 0.4[b]		14.2 ± 1.1		9.5 ± 0.9		46.3 ± 1.3	74.2 ± 1.7	24.8 ± 1.4

[a] Free and bound polysomes were isolated from 25% homogenate of rat liver and purified by centrifugation through discontinuous gradients in the absence of cell sap as described under Methods. The RNA content of the heavy-sucrose layer and pellet was determined as described under Methods. [b] Mean ± standard error.

brane-bound RNase activity is not inhibited by cell sap or is not removed from the bound polysomes during the usual purification procedure, or both.

To investigate the first possibility, pellets resulting from the first centrifugation were suspended in cell sap containing KCl to a final concentration of 75 to 500 mM and then treated to remove nuclei. Rahman (1966) has demonstrated that concentrations of KCl ranging from 250 to 500 mM inhibit cell sap insensitive alkaline RNase activity in the particulate fraction by 80–100%. The supernatants remaining after detergent treatment were centrifuged on sucrose gradients to display the polysomes. Comparison of the absorbance profiles (not shown) revealed that the polysomes were partially degraded at concentrations of KCl below 250 mM. However, the magnitude of this effect was not sufficient to account for the extent of breakdown observed after purification (Figure 2a); hence, breakdown must also occur during the purification step.

To test this inference, polysome purification was studied in the presence of 250 and 75 mM KCl, and with or without cell sap. Typical profiles are presented in Figures 1 and 2, and quantitative data from three experiments comparing all four conditions are summarized in Table I. The results (Table I) show that the average size of both classes of polysomes was significantly larger in the preparations purified through 250 mM KCl compared to that purified through 75 mM KCl, and that cell sap had little effect at either KCl concentration. After purification at 250 mM KCl, 84% of the free and 90% of the bound ribosomes sedimented in the polysome region of the gradient with the largest proportion (50%) in the region that corresponds to polysomes containing more than eight ribosomes. Additional peaks with intermediate sedimentation coefficients (such as 1½-somes) were more prominent in polysomes purified through 250 mM KCl; they may represent initiation complexes (Hoerz and McCarty, 1969). In the experiments which follow, 250 mM KCl–cell sap was used to isolate the bound fraction and to purify both classes of polysomes.

Distribution and Recovery of Polysomes. To ascertain whether sedimentation of polysomes and ribosomes was complete, the heavy-sucrose layer of the discontinuous gradients was subjected to RNA and sedimentation analysis. Table II shows that 23% of the free and 17% of the bound ribosomes were recovered in the 2.0 M layer. Sedimentation analysis revealed that these fractions were comprised mainly of subunits and monosomes. Of the total homogenate RNA, 74% was recovered in the form of ribosomes. Considering the loss of about 5% of total rRNA (1–2% in the nuclear pellet and 3% as rough microsomes in the purification of free polysomes) this yield is comparable to previous estimates based only on pelleted ribosomes (Blobel and Potter, 1967). Approximately 19% of homogenate RNA was recovered in the form of free and 56% in the form of bound ribosomes; hence, free and bound ribosomes comprise 25 and 75%, respectively, of total rRNA, a result consistent with that obtained with a different cell fractionation scheme (Blobel and Potter, 1967).

Cross-Contamination between Free and Bound Polysomes. To quantitate the specificity of the method for separating free and bound polysomes, [^{14}C]orotate-labeled free

Table III: Analysis of Cross-Contamination between Free and Membrane-Bound Polysome Fractions.[a]

Fraction	^{14}C-Free Polysomes		^{14}C-Rough Microsomes	
	dpm/ml of Homogenate	%	dpm/ml of Homogenate	%
Free supernatant				
1. Light layers				
(a) Acid soluble	300	0.9	100	0.2
(b) Acid insoluble	700	2.2	2 500	4.9
2. Heavy layer				
(a) Acid soluble	100	0.3	100	0.2
(b) Acid insoluble	5 000	15.4	1 000	2.0
3. Pellet	22 300	68.8	1 400	2.8
		87.6[b]		10.1
Bound supernatant				
1. Light layers				
(a) Acid soluble	100	0.3	200	0.4
(b) Acid insoluble	200	0.6	1 100	2.2
2. Heavy layer				
(a) Acid soluble	100	0.3	100	0.2
(b) Acid insoluble	700	2.2	9 000	17.8
3. Pellet	2 900	9.0	35 100	69.4
		12.4		90.0

[a] ^{14}C-free polysomes and -rough microsomes (526 000 dpm/mg of RNA) were prepared from rat liver 6 days after injection of [6-^{14}C]orotate and purified; aliquots were added to portions of 25% homogenate, and free and bound polysomes were prepared from it as described under Methods. Radioactivity among the various fractions of the discontinuous gradients was determined as described under Methods. The values shown represent the mean of two separate experiments. [b] Percent recovery of total disintegrations per minute (range 98–100%).

polysomes and rough microsomes were prepared as described above. With ^{14}C-free polysomes, 85% of the radioactivity was recovered in the free and only 11% in the bound fraction (Table III), indicating that very few free polysomes were pelleted during the first centrifugation, since the pellet comprised 14% of total homogenate volume. With ^{14}C-labeled rough microsomes, 87% of the radioactivity was recovered in the bound and 5% in the free fraction. However, the latter is probably an overestimate, since an almost identical amount of radioactivity could be pelleted by centrifuging the ^{14}C-labeled rough microsomes directly on a discontinuous gradient, suggesting that they were contaminated with free polysomes even after extensive washing. Combining this information with estimates of the distribution of polysomal RNA (Table II), we conclude that there is about 3% contamination of bound polysomes with free and less than 1% contamination of free polysomes with bound.

Activity of Polysomes. To ascertain whether purification conditions affect polysome activity, polysome preparations (Figures 1 and 2) were incubated with labeled amino acids and incorporation into polypeptides was determined. Figure 3a shows that the rate of incorporation was linear over the first 4 min and approximately the same for all four free preparations, although polysomes purified through cell sap–250 mM KCl gradients had the highest rate at all intervals. Similarly, bound polysomes purified under the same conditions had the highest rate, and the only rate that was linear for 4 min and comparable to that of the free polysomes (Figure 3b). The differences between bound polysomes purified in the presence and absence of cell sap can be accounted for by the fact that cell sap contains factors that were removed by detergents during the isolation step. In contrast, the low activity observed after purification at 75 mM KCl was probably due to polysome breakdown. However, that the correspondence in the activity of the two classes of polysomes is not an artifact due to differential contamination with either deoxycholate, membranes, glycogen, or RNase is indicated below.

Characterization of the Polypeptides Synthesized in Vitro. Before proceeding with the analysis of the polypeptides synthesized in vitro, it is essential to establish whether polysomes can be prepared in an undegraded state and whether they are contaminated with completed proteins. Rats were pulse labeled with [^{3}H]leucine, and the polysomes were prepared and displayed on sucrose gradients. Figures 1 and 2 show the distribution of radioactivity across the gradients. The amount of radioactivity in the monosome and supernatant region of the free polysomes was small whether they were prepared in the presence of 75 or 250 mM KCl (Figures 1a and 1c). Similar results could be obtained with bound polysomes only if they were prepared in the presence of 250 mM KCl (cf. Figures 2a and 2c). Thus, there appears to be very little polysome breakdown during the isolation and purification steps, and little contamination with completed proteins. Moreover, after EDTA treatment, which dissociates the polysomes into subunits, essentially all the radioactivity sedimented as a single peak in the supernatant region, indicating that ribosomal proteins were not labeled. Thus, the radioactivity was almost exclusively in nascent polypeptide chains.

To understand better the properties of the liver cell-free system and to compare the products synthesized by free and bound polysomes, nascent polypeptides were labeled in vivo from a large pool of low specific activity [^{3}H]leucine (Loftfield and Harris, 1956) prior to incubation of the polysomes with [^{14}C]leucine, and then the released polypeptides were isolated and subjected to sodium dodecyl sulfate–acrylamide gel electrophoresis (Figure 4). In this experiment, 54 and 39% of total polypeptides were released by free and bound polysomes, respectively, and in several such experiments the same trend has been observed. Released polypeptides migrated as numerous peaks over a wide range of mo-

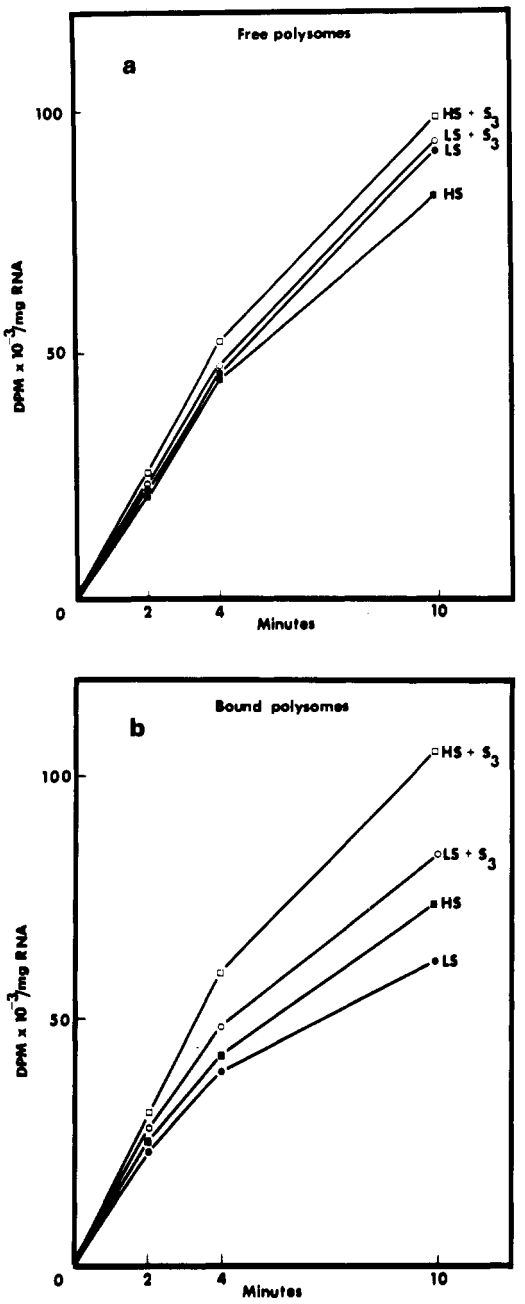

FIGURE 3: Time course of ^{14}C-labeled amino acid incorporation in vitro by free (a) and bound (b) polysomes. A second series of aliquots of the preparations depicted in Figures 1 and 2 was incubated (4.8 A_{260} units/ml) with a mixture of radioactive amino acids in a cell-free system and hot acid-insoluble radioactivity was measured as described under Methods: polysomes purified in the presence of 75 mM KCl (LS) with (○) and without (●) cell sap (S_3); polysomes purified in the presence of 250 mM KCl (HS) with (□) and without (■) cell sap.

lecular weight; ^3H and ^{14}C peaks coincided closely, suggesting that only completed polypeptides were released. The isotopic ratio in the polypeptides (Figure 4, upper curve) declined sharply with increasing molecular weight, indicating that the extent of release fell off rapidly with increasing polysome size. Since this ratio rarely exceeded that expected for one round of translation, little, if any, reinitiation occurred. The slope of the ratio curve in the two profiles was virtually identical, indicating that polypeptides of various size classes were released to the same extent by both free and bound polysomes. However, the profiles were clearly different.

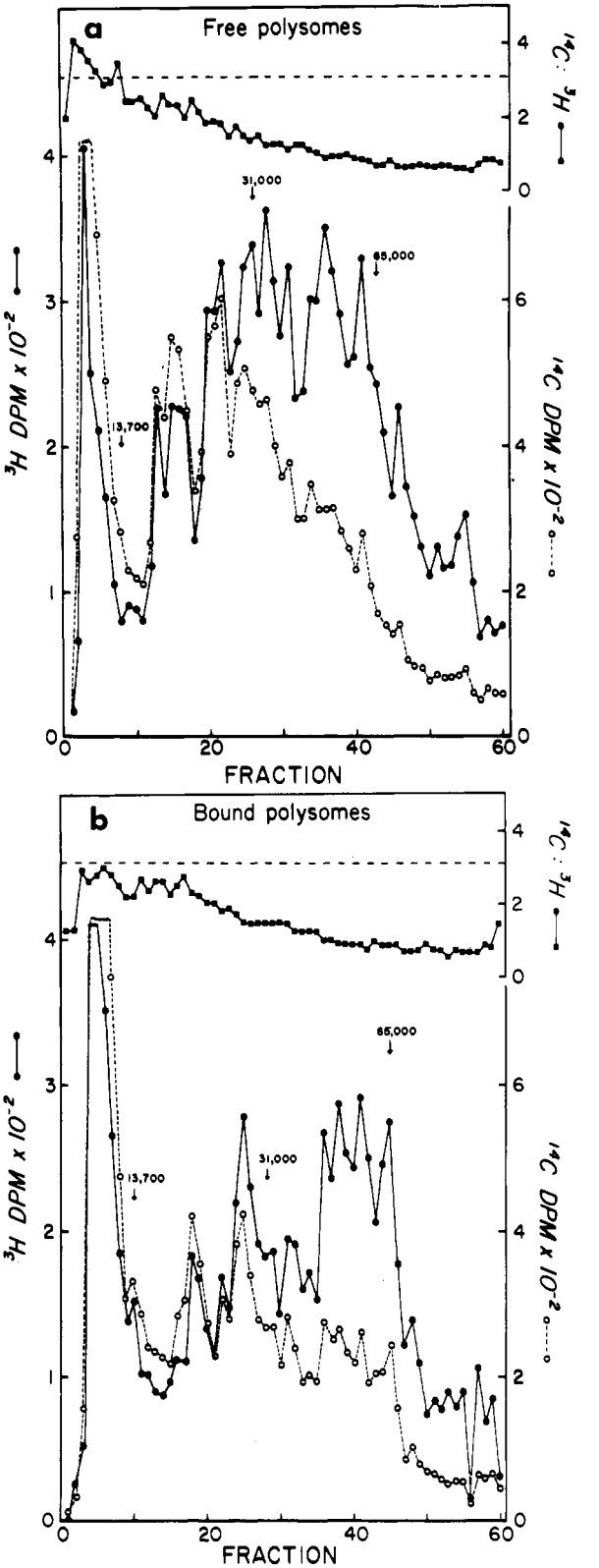

FIGURE 4: Molecular weight profiles of polypeptides released from free (a) and bound polysomes (b) in vitro. Nascent polypeptides were labeled in vivo with [^3H]leucine (25 mCi/kg body weight, 100 mCi/mmol) for 5 min and polysomes were prepared and incubated (2.3 A_{260} units/ml) for 1 h with [^{14}C]leucine in a cell-free system as described under Methods; after centrifugation to remove ribosomes, the released polypeptides were isolated and subjected to sodium dodecyl sulfate–acrylamide gel electrophoresis (1 mg of protein/gel) as described. The migration of ribonuclease (mol wt 13 700), deoxyribonuclease (mol wt 31 000), and bovine serum albumin (mol wt 65 000) on similar gels is indicated: ^3H incorporated into nascent chains (●); ^{14}C incorporated into released polypeptides (○); ratio $^{14}C/^3H$ in released polypeptides (■); ratio $^{14}C/^3H$ presumed for one round of translation (- - -).

Table IV: Glycogen and Phospholipid Content of Free and Bound Polysomes.[a]

	Expt	RNA μg/g of Liver	Glycogen μg/g of Liver	Glycogen/ RNA	Phospholipid[b] μg/g of Liver	Phospholipid/ RNA
Homogenate	1	9230	520	0.06	23 500	2.55
	2	8270	210	0.02	19 700	2.38
				0.04 ± 0.02[c]		2.46 ± 0.08
Free polysomes	1	1900	13	0.01	18	0.01
	2	1840	38	0.02	14	0.01
				0.02 ± 0.01		0.01 ± 0.00
Bound polysomes	1	5970	30	0.01	78	0.01
	2	5410	56	0.01	86	0.02
				0.01 ± 0.00		0.02 ± 0.01

[a] Free and bound polysomes were prepared from a 25% homogenate of liver of rats fasted for 16–18 h. RNA, glycogen, and phospholipid phosphorus were determined as described under Methods. [b] The phospholipid value was obtained by multiplying the phosphorus value by 25. [c] Mean ± standard error.

Table V: Distribution of [Me-^{3}H]Choline-Labeled Membrane Phospholipid in Free and Bound Polysomes.[a]

	Acid-Insoluble Radioact.		Acid-Insoluble, Lipid Extractable Radioact.		
	dpm/g of Liver	%	dpm/g of Liver	%	dpm/mg of RNA
Homogenate	974 000	100.00	970 000	99.60	116 000
Free polysomes	708	0.07	648	0.07	460
Deoxycholate-treated free polysomes	48	0.00	20	0.00	15
Bound polysomes	372	0.04	340	0.04	360

[a] Rats (starved 16–18 h) were injected with 0.5 ml of [Me-^{3}H]choline (50 μCi/100 g body weight, 2.34 Ci/mmol) containing 10 mg of Bactocasamino acids and killed 30 min later. Free and bound polysomes were prepared from 25% homogenates; total and lipid extractable trichloroacetic acid insoluble radioactivity was determined as described under Methods.

Analysis of Polysome Contaminants. To determine whether deoxycholate sediments along with bound polysomes, the supernatant remaining after removal of nuclei was treated with [^{14}C]deoxycholate and bound polysomes were harvested. Of the total deoxycholate, only 0.05% was recovered in bound polysomes. The deoxycholate to RNA ratio was 0.008, indicating that the extent of contamination is well below that observed by Korner (1961) to interfere with cell-free protein synthesis.

Table IV summarizes the results of glycogen and phospholipid analysis. After purification, glycogen and phospholipid to RNA ratios of both classes of polysomes ranged from 0.01 to 0.02; they are substantially lower than previously reported for rat liver polysomes (Bloemendal et al., 1967; Murty and Hallinan, 1969; Andrews and Tata, 1971).

To validate the phospholipid analyses, rats were pulse labeled with [Me-^{3}H]choline for 30 min, and polysomes were prepared and assayed for radioactivity. Nagley and Hallinan (1968) have demonstrated that with a pulse of this duration, membrane lecithin and sphingomyelin but not other cellular constituents are labeled. Of the total lipid extractable radioactivity (disintegrations per minute per gram of liver) only 0.07 and 0.04% were found in free and bound polysomes, respectively (Table V). The phospholipid specific activity (disintegrations per minute per milligram of RNA) was 250- to 320-fold lower in the polysomes compared to the homogenate, indicating that the extent of purification is comparable to that observed with phospholipid analysis (Table IV). However, in contrast to the findings for bound polysomes, deoxycholate treatment completely eliminated membrane contamination of the free polysomes.

The possibility that comparable rates of amino acid incorporation into protein by free and bound polysomes might result from differential RNase contamination was also examined. To test for such activity, polysome preparations were incubated either at 0 or 37 °C for 15 min and then displayed on sucrose gradients. The results showed that the polysomes incubated at 37 °C had essentially the same profile as those incubated at 0 °C.

Discussion

The method developed in this laboratory for separating free and bound polysomes has several advantages over other methods used for this purpose. It is rapid and reproducible, requires much less ultracentrifugation than the isopycnic technique, and provides a nearly quantitative means of separating the two classes of polysomes (Venkatesan and Steele, 1972). Moreover, it can now be employed to obtain polysomes that appear to be undegraded, biologically active, and reasonably free of contaminants.

The procedure described here differs from the initial procedure in several important respects. The particulate fraction resulting from the first centrifugation of the homogenate is suspended in cell sap–250 mM KCl rather than cell sap before detergent treatment. This has two effects. It protects bound polysomes from degradation by cell sap sensitive as well as cell sap insensitive alkaline RNase activity during the solubilization of the rough microsomes without lysing the nuclei and releasing their ribonucleoprotein complexes and DNA. Secondly, the polysomes are separated from the nonribosomal components of the cell by pelleting

them through sucrose gradients containing cell sap and 250 mM KCl rather than polysome buffer. This also has two effects. The moderate concentration of KCl prevents differential breakdown of bound polysomes during the purification step, due to inhibition of RNase activity and elimination of polysomal binding of RNase. Cell sap, on the other hand, elevates the protein-synthesizing activity of bound polysomes to that of free polysomes, presumably by replacing or restoring the activity of factors that were lost during the isolation step. The treatment of cells and tissues with hypertonic buffers has been reported both as a method of improving polysome yield (Heywood et al., 1967; Gielkens et al., 1971; Jernigan et al., 1972) and for suppressing RNase activity (Earl and Morgan, 1968; Ascione and Arlinghaus, 1970); however, until now, no method has been available for isolating undegraded bound polysomes from the sedimentable fraction of rat liver. By comparison, combinations of cell sap and high concentrations of heparin (Palmiter, 1974) could not prevent bound polysome breakdown, presumably because heparin acts as a competitive inhibitor of acid rather than alkaline RNase activity (Shortman, 1962).

The integrity of the polysomes can be evaluated from both a structural and a functional standpoint provided that the populations studied are representative. The procedure described here gives nearly quantitative recovery of polysomes. Moreover, it enables the isolation of polysomes in a relatively undegraded state as judged by their size distribution on sedimentation analysis, their low content of labeled monosomes after pulse labeling with [^{3}H]leucine in vivo, and their ability to complete and release a spectrum of polypeptides that is in accord with that observed for liver proteins (Schnaitman, 1969).

The purity of the polysomes can be assessed by chemical and isotopic methods. The major possible contaminants are membranous material and glycogen. The purification procedure described here removes virtually all of these contaminants. Free polysomes, for example, have a phospholipid to RNA ratio of 0.008. This may be compared with the results of Bloemendal et al. (1967), Murty and Hallinan (1969), and Andrews and Tata (1971), who obtained ratios of 0.021, 0.160, and 0.048, respectively, with conventional purification procedures.

More important for subsequent studies is the contamination between classes of polysomes, for if one wishes to study functional specialization, valid estimates of the degree of cross-contamination must necessarily be known to avoid misinterpretations. The specificity of the method for separating free and bound polysomes has been evaluated by both isotopic (the present study) and chemical techniques (Venkatesan and Steele, 1972). The degree of cross-contamination is routinely low, making the level of contamination less than 3% of total RNA of each class. The method is potentially useful for other tissues, but it may be less satisfactory for the isolation of bound polysomes from tissues with much less rough endoplasmic reticulum than liver because of increased contamination of bound polysomes with free polysomes.

The quantitative isolation of homogeneous, undegraded populations of polysomes should facilitate the analysis of the mechanisms by which a variety of factors influence protein synthesis in the liver. It could also facilitate the identification of factors involved in ribosome segregation as well as the analysis of functional specialization in conjunction with immunological techniques.

There is substantial evidence that the two classes of liver polysomes are engaged in the synthesis of different sets of proteins (Ganoza and Williams, 1969; Hicks et al., 1969; Redman, 1969). Hence, a crucial test of the specificity of the method for separating free and bound polysomes is the demonstration of different populations of polypeptides. The finding of quantitative, and possibly qualitative, differences in the molecular weight distribution of polypeptides released by free and bound polysomes in vitro, while not providing definitive proof, is consistent with this criterion.

Acknowledgments

The authors would like to thank Ms. Linda Kessler and Mr. Wayne Patton for expert technical assistance.

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Induction of Stable Protein–Deoxyribonucleic Acid Adducts in Chinese Hamster Cell Chromatin by Ultraviolet Light†

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ABSTRACT: Ultraviolet (uv)-light-mediated formation of protein–DNA adducts in Chinese hamster cell chromatin was investigated in an attempt to compare chromatin alterations induced in vitro with those observed in vivo. Three independent methods of analysis indicated stable protein–DNA associations: (1) a membrane filter assay which retained DNA on the filter in the presence of high salt–detergent; (2) a Sepharose 4B column assay in which protein eluted coincident with DNA; and (3) a CsCl density gradient equilibrium assay which showed both protein and DNA banding at densities other than their respective native densities. Treatment of the irradiated chromatin with DNase provided further evidence that protein–DNA and not protein–protein adducts were being observed in the column assay. There is a fluence-dependent response of protein–DNA adduct formation when the chromatin is irradiated at low ionic strength and is linear for protein over the range studied. When the chromatin is exposed to differing conditions of pH, ionic strength, or divalent metal ion concentration, the quantity of adduct formed upon uv irradiation varies. Susceptibility to adduct formation can be partially explained in terms of the condensation state of the chromatin and other factors such as rearrangement, denaturation, and dissociation of the chromatin components. Besides providing information on the biological significance of these types of uv-induced lesions, this technique may be useful as a probe of chromatin structure.

A variety of photoproducts can be induced in cells upon exposure to ultraviolet (uv) light. Although it has been shown that pyrimidine dimers in DNA can lead to serious biological consequences, including cell death, the biological significances of other photo-induced products in the cell are relatively unknown. Previous studies with procaryotic or eucaryotic cells have demonstrated a decrease in extractability of DNA with increasing uv light fluences (Alexander and Moroson, 1962; Smith, 1962). This phenomenon was attributed to the formation of stable protein–DNA adducts. More recently, in an attempt to understand the photochemistry of these protein–DNA complexes, various workers have demonstrated the covalent linkage of amino acid molecules to the free bases of DNA, to synthetic polynucleotides, and to native DNA (Smith and Aplin, 1966; Smith and Meun, 1968; Smith, 1969, 1970; Varghese, 1973; Schott and Shetlar, 1974). Furthermore, it has been shown that a variety of protein molecules, including bovine serum albumin, DNA and RNA polymerases, aminoacyl tRNA synthetases, and ribosomal proteins, can be linked to nucleic acids via uv light (Smith, 1964; Markovitz, 1972; Schoemaker and Schimmel, 1974; Strniste and Smith, 1974; Gorelic, 1975). The biological consequences of uv-induced protein–DNA adducts are not well understood; yet, under certain experimental conditions, it has been reported that this type of lesion can be correlated with lethality in bacteria (Ashwood-Smith et al., 1965; Smith and O'Leary, 1967). Several other lines of evidence also indicate the possible involvement of proteins in the modification or inactivation of eucaryotic cells by uv light (Chu, 1965; Todd et al., 1968; Habazin and Han, 1970; Han et al., 1975).

The chromatin of eucaryotic cells is a heterogeneous complex of DNA with acidic (nonhistone) and basic (histone) nuclear proteins. Since it has been shown that uv-light irradiation of mammalian cells induces cross-links between protein and DNA (Alexander and Moroson, 1962; Habazin and Han, 1970; Han et al., 1975), this investigation was initiated to determine the possible role of chromosomal proteins in the cross-linking process. Using chromatin isolated from Chinese hamster cells and three independent methods of analysis, the results show a fluence-dependent relationship in the protein–DNA adducts formed. The degree of cross-linking is dependent upon the ionic environment of the

† From the Cellular and Molecular Biology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87545. *Received August 28, 1975.* This work was performed under the auspices of the U.S. Energy Research and Development Administration. S.C.R. was a postdoctoral appointee at the Los Alamos Scientific Laboratory for the duration of these studies.