Chromatographic Isolation of 80S Ribosomes from Rat Liver and Mouse Plasma Cell Tumor*

Elbert A. Peterson and Edward L. Kuff

ABSTRACT: The isolation of ribosomes from rat liver and mouse plasma cell tumor was achieved with the aid of a new adsorbent (ECTHAM-cellulose) specifically designed to permit their elution by dilute salt solutions. Microsomes, free ribosomes, and ribonucleic acid were adsorbed under conditions that allowed nearly all other components of the homogenate fraction to pass through; then microsomal lipoprotein was removed by passing a nonionic detergent through the column.

A subsequent gradient of NaCl eluted ribosomes in two major peaks. In the case of rat liver, the ribosomes in these two fractions differed in sedimentation rate (83 and 77 S) and in protein:ribonucleic acid ratio (2.4 and 1.9). Both contained significantly more protein than ribosomes prepared by conventional means with deoxycholate. On rechromatography or storage at 0°, the ribosomes in the first peak shifted to the position of the second. Changes in the magnesium concentration altered the chromatographic behavior of the ribosomes. Labeling the ribonucleic acid of plasma cell tumors *in vivo* with [^{3}H]uridine resulted in chromatographic fractions having different specific activities. The most firmly bound fraction contained ribonucleic acid of very high specific activity.

Ribosomes of *Escherichia coli* have been purified by chromatography on DEAE-cellulose (Bolton *et al.*, 1959; Salas *et al.*, 1965; Furano, 1966; Stanley *et al.*, 1966; Furano and Hollis, 1967). However, elution from this adsorbent has generally required salt concentration (0.4 M or greater of sodium, potassium, or ammonium chloride) sufficient to dissociate the ribosomes to 30S and 50S subunits. Fortunately, these subunits can be efficiently recombined to form active 70S ribosomes, even though the chromatographed and reassociated preparations have lost about one-third of their original protein content, and such partially stripped ribosomes have been of considerable value in the investigation of ribosomal function.

To our knowledge, however, chromatographic procedures have not hitherto been successfully applied to the isolation and purification of mammalian ribosomes, which appear to be more labile than those of bacterial origin. The work to be presented here has achieved some progress in that direction by utilizing an adsorbent specifically designed to have an affinity for ribosomes sufficiently low to permit their elution in undissociated form by dilute salt solutions. Moreover, the procedures include a novel method for preparing mammalian ribosomes free from any microsomal membranes to which they may have been attached. Such chromatographically isolated ribosomes are active in amino acid incorporation when supplemented with soluble factors, energy source, and synthetic polyribonucleotide, as described in an accompanying report (Kedes *et al.*, 1969).

Experimental Procedures

Preparation of Homogenate Fractions. Livers were obtained from male Sprague–Dawley rats weighing between 250 and 325 g and fed *ad libitum* until sacrificed. The excised organs were cooled to 0°, minced, and homogenized for 2 min in cold 0.25 M sucrose (3.0 ml/g of liver), using a motor-driven pestle of the Potter-Elvehjem type.

Nuclei and mitochondria were sedimented as a combined fraction by centrifugation of the homogenates for 10 min at 5000*g* (Schneider and Hogeboom, 1950). The pellets were resuspended in fresh 0.25 M sucrose (1.5 ml/g of liver) by brief homogenization and resedimented at 10,000*g* for 10 min. The combined supernatant fluids of these centrifugations, made to a final volume of 5.0 ml/g of liver, constituted the mitochondrial supernatant fraction.

Microsome pellets were obtained by centrifugation of the mitochondrial supernatant fractions for 90 min at 105,000*g* (rotor no. 40, Spinco Model L ultracentrifuge). The pellets were resuspended by homogenization in 0.25 M sucrose–5 mM Tris-HCl–0.5 mM $MgCl_2$ (pH 7.4; 2.5 ml/g of liver).

For some experiments, ribosomes were prepared from resuspended microsomes through the use of deoxycholate as previously described (Kuff and Zeigel, 1960), with the exception that 5 mM Tris-HCl–0.5 mM $MgCl_2$ (pH 7.4) was substituted for the dilute phosphate–magnesium buffer originally employed.

Microsomes were prepared from subcutaneously growing RPC-20 plasma cell tumor as described in Kuff and Hymer (1966), except that two volumes of 0.25 M sucrose–1 mM $MgCl_2$ was used for homogenization instead of four volumes of 0.25 M sucrose–4 mM $MgCl_2$. The animal was decapitated and the tumor removed 1 hr after intraperitoneal injection of 80 μCi of [^{3}H]uridine contained in 0.9 ml of sterile saline solution (specific activity 2 mCi/μmole, New England Nuclear Corp.).

Homogenate fractions were applied to the column as soon as possible after their preparation, unless otherwise stated. All operations were carried out at *ca.* 5°.

Preparation of ECTHAM-Cellulose.[1] Tris (20 g; Sigma

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received July 31, 1967.* Presented in part at the 45th Annual Meeting of the American Society of Biological Chemists, Atlantic City, N. J., 1961.

[1] This adsorbent was named by combining letters selected from the names of the reactants, epichlorohydrin and Tris.

7–9) was dissolved in a solution of 60 g of NaOH in 175 ml of water. The resulting solution was chilled in an ice bath and then stirred into 60 g of cellulose (100–230 mesh, sieved from Solka-Floc SW-40-B obtained from the Brown Co., Berlin, N. H.), mixing well with a strong stirring rod. The dry, crumbly mixture was immersed in an ice bath for 30 min and stirred occasionally. Then 30 ml of epichlorohydrin (Eastman Organic Chemicals) was added in small portions and mixed in thoroughly. The vessel was covered with Parafilm and allowed to stand at room temperature overnight in a hood. The mixture gradually rose in temperature and became perceptibly warm, reaching a maximum temperature 2–3 hr after addition of the epichlorohydrin.

To the dry, crumbly product, 500 ml of 2 M NaCl was added, then most of the liquid was removed from the soft mass by gentle suction on a coarse-sintered glass filter. The filter cake was washed successively with 1 N NaOH, 1 N HCl, and 1 N NaOH, with water rinses after each reagent wash.

The modified cellulose was then diluted to 8–10 l. with water and washed repeatedly by decantation to remove fines. It was finally filtered, washed thoroughly with ethanol (without disrupting the filter cake), and pulled dry on the filter. The remaining ethanol was removed *in vacuo* in a rotating evaporator fitted with a coarse-sintered glass filter and warmed by a 40° water bath; 1 hr of tumbling yielded about 55 g of dry adsorbent having a nitrogen content corresponding to 0.14–0.16 mequiv/g.

Chromatographic Procedures. ECTHAM-cellulose (10 g) was allowed to sink into approximately 150 ml of 0.5 N NaOH to accelerate equilibration of the dried material with water. After a few minutes the suspension was diluted severalfold with water and allowed to settle. Any "fines" in the supernatant fluid were discarded, and the sediment was transferred to a coarse-sintered glass funnel and washed free of alkali. It was then conditioned with the starting buffer by washing twice with 100-ml portions and finally resuspended in about 500 ml of starting buffer. This suspension was poured into a glass or Lucite column (1.8-cm i.d.) fitted with a 500-ml conical reservoir (Peterson and Sober, 1962). Low air pressure was applied immediately and gradually raised (in proportion to the rising height of the packed material) from about 5 psi at a column height of 4 cm to 15 psi at the final height of about 24 cm.

The washing of the adsorbent and the packing of the column were carried out at room temperature. The column was then transferred to a cold room (4–5°), where it was washed with an additional amount of cold starting buffer. The very low base content of the adsorbent made equilibration with the starting buffer an easy matter; the final washing of the finished column at the temperature that was to be used for chromatography achieved this with a reasonable volume of buffer.

Columns thus prepared could be used repeatedly without repacking. Since the chromatographic experiments were always terminated by an elution with 0.1 M Na_3PO_4, it was only necessary to flush out the remaining Na_3PO_4 with water and then pump through 200–300 ml of starting buffer to bring the pH and conductivity of the effluent to the desired levels for reuse.

A column containing about 8 g of Sephadex G-50 equilibrated with starting buffer was used for buffer exchange in experiments employing mitochondrial or particle-free supernatant fluid. The Sephadex column was mounted above the ECTHAM-cellulose column and connected to it with small tubing. The sample of supernatant fluid (10 ml) was then applied to the Sephadex column, and its progress was followed by observing the color on the column, and the turbidity in the connecting tubing. When the last of the sample had passed into the ECTHAM-cellulose and before the salt front could reach it, the Sephadex column was disconnected, and starting buffer was pumped directly into the ECTHAM-cellulose column to wash out the unadsorbed components. In later work it was found that dilution of the mitochondrial or particle-free supernatant fluid with an equal volume of water permitted direct application to the ECTHAM-cellulose, making the Sephadex column unnecessary. In experiments employing microsome pellets, the resuspended microsomes were also applied directly, since they were resuspended in the starting buffer.

In nearly all of the experiments to be described, the starting buffer was 5 mM in chloride, 6.25 mM in Tris, and 0.5 mM in $MgCl_2$. It was prepared by mixing 31.2 ml of 0.40 M Tris, 10.0 ml of 1.00 N HCl, and 10.0 ml of 0.10 $MgCl_2$, then diluting the mixture to 2000 ml with water. The pH varied from 7.4 to 7.5, depending upon the room temperature at which it was measured.

The same gradient of NaCl concentration was used in every experiment reported here, although numerous variations in the accompanying $MgCl_2$ were made. The NaCl rose from a concentration of zero (starting buffer) to a limit value, L, of 2.0 M, following a curve in which $C = (v/V)^4L$. This was accomplished by using five chambers of a Varigrad (Peterson and Sober, 1959) with the limit solution in the fifth chamber and starting buffer in the other four. Each chamber initially contained 100 g of liquid. The standard limit solution was 2.0 M NaCl–5 mM Tris HCl–20 mM $MgCl_2$ (pH 7.6). As in the starting buffer, the molar ratio of Tris to HCl was 1.25.

Triton X-100, a nonionic detergent, was obtained from Z. D. Gilman, Inc., Washington, D. C., and was used without further purification. A 10% solution in water was prepared, and small portions of this were diluted with starting buffer to the desired concentration (0.9%). The use of larger amounts and higher concentrations of the detergent than specified in the experiments described herein did not change the results.

Examination of Effluent. The fractions collected were examined for absorbance at 260, 280, and 415 mμ, providing convenient indices of nucleoprotein and heme-bearing microsomal protein. The pH and conductivity of each fraction were measured at room temperature.

Ultracentrifugal analysis of selected fractions was carried out at 20° in a Spinco Model E ultracentrifuge equipped with an ultraviolet absorption system. Film densities were scanned and plotted by means of a Spinco Model R Analytrol with a microanalyzer attachment.

RNA was determined by precipitation from the tissue preparations and effluent fractions with 6% perchloric acid at 0°. The precipitates were washed with cold 5% perchloric acid, the lipids were extracted, and the residues were finally heated for 15 min at 70° in 6% perchloric acid (Littlefield *et al.*, 1955). The absorbance of the hot perchloric acid extract was expressed in terms of RNA phosphorus (RNA-P) through the use of an experimentally determined extinction coefficient, $E_{260}^{1\,cm}$, of 0.342/μg of RNA-P. Total nitrogen was measured by nesslerization of acid digests. Protein was determined with the Lowry–Folin procedure (Lowry *et al.*, 1951), using crystallized bovine serum albumin (Armour and Co.) as a reference standard. The RNA was acid precipitated and prepared

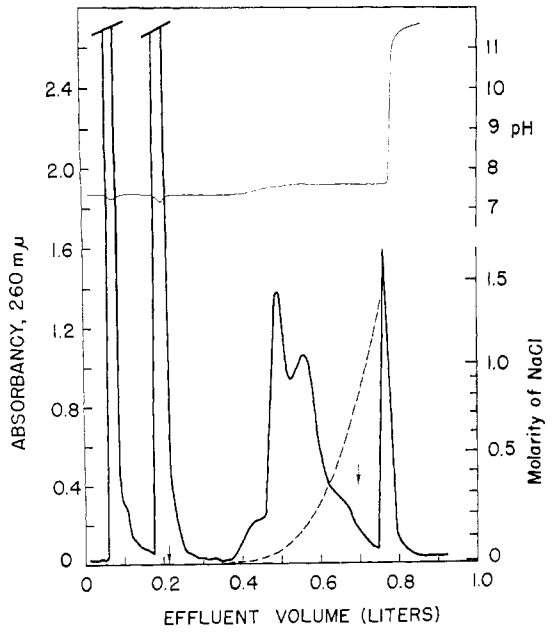

FIGURE 1: Elution pattern obtained when 10 ml of mitochondrial supernatant fraction corresponding to about 3 g of rat liver and equilibrated with starting buffer (5 mM Tris-HCl–0.5 mM $MgCl_2$, pH 7.5) by passage through Sephadex G-50 was applied to a 24 × 1.8 cm i.d. column containing 10 g of ECTHAM-cellulose (0.16 mequiv of N/g) equilibrated with the same buffer. At about 0.13 l., 16 ml of 0.9% Triton X-100 was introduced and washed through with starting buffer. Gradient to 2.0 M NaCl–5 mM Tris-HCl–20 mM $MgCl_2$ (pH 7.6) started at the point marked by first arrow (0.22 l.). Last arrow marks point at which 0.1 M Na_3PO_4 was introduced. The flow rate was 150 ml/hr and the temperature was 5°. Heavy solid line represents absorbancy of effluent at 260 mμ; broken line indicates salt concentrations measured and plotted as conductivity but translated into molarity of NaCl in scale at the right. Fine line in upper part of the figure shows the pH of the effluent. The first peak is unadsorbed material; the second is the Triton wash. RNP-I and RNP-II emerged at 0.49 and 0.56 l., respectively.

for assay of radioactivity as described elsewhere (Kuff and Hymer, 1966).

Exposure of tissue fractions to Triton X-100 was found to interfere seriously with the subsequent analysis for lipid phosphorus (lipid-P) when acid precipitation steps were employed in the analytical procedure; phospholipids (but not RNA) were rendered partly acid soluble in the presence of the detergent. Accordingly, lipids were extracted directly from the fractions with methanol and chloroform as follows: 5 ml of each solvent was added in the above order to 1 ml of fraction, and the resultant solution was warmed to 55° for 5 min. The precipitate was removed by centrifugation and discarded. A clear chloroform phase was obtained by the addition of 4 ml of 0.5 M NaCl to the mixture, followed by centrifugation. The chloroform was evaporated to dryness, the lipids were digested with sulfuric acid, and phosphorus was determined by the method of Fiske and Subbarow (1925). Recovery of lipid-P by this method was quantitative, even in the presence of Triton X-100 in concentrations up to 2%.

The volume of each fraction was marked on the side of the tube when it was removed from the collector. After the tubes had been emptied, they were rinsed and dried, then refilled to the mark using a self-filling buret. The cumulative volumes thus obtained were used in plotting the figures.

Results

Ribosomes from Mitochondrial Supernatant Fraction. Figure 1 illustrates the results obtained when a mitochondrial supernatant fraction corresponding to about 3 g of rat liver was applied to a column containing 10 g of ECTHAM-cellulose and chromatographed under the conditions that will herein be designated as "standard." Replacement of the small molecules of the homogenate with those of the starting buffer was accomplished by passing the sample through Sephadex G-50 before it entered the adsorbent column. Nearly all of the microsomes were adsorbed on the ECTHAM-cellulose; however, glycogen particles, microsomes low in RNA content, and the great bulk of the soluble protein passed through and appeared in the first peak of Figure 1. This "pass-through" fraction was characterized by obvious turbidity, as well as a pink color which was reflected in a high absorbancy at 415 mμ. Subsequent treatment of the column with a solution of Triton X-100 stripped the microsomal lipoprotein from the ribosomes, leaving the latter adsorbed to the column, along with the tRNA that was introduced with the sample. The microsomal protein removed by the Triton X-100 emerged from the column in the second peak (at 0.2 l.), which also was visibly colored as a result of its content of microsomal cytochrome components. Most of the ultraviolet absorption of this peak was due to the Triton, however.

Starting at the point marked by the first arrow in the figure, a gradient of NaCl was superimposed on the Tris-HCl buffer. A gradient of $MgCl_2$ accompanied the NaCl, the ratio of the Na and Mg increments being 100:1. Two major peaks with a high A_{260}:A_{280} ratio (about 1.8) were eluted by the salt gradient, emerging at 0.04 and 0.14 M NaCl, respectively. Upon examination in the analytical ultracentrifuge, the material in the first peak (RNP-I, at 0.49 l.) was found to consist largely of 83S nucleoprotein particles, accompanied by a more slowly sedimenting component that proved to be tRNA (see below). The second peak (RNP-II, at 0.56 liter) contained 77S particles and was almost free of smaller material. The material in the small shoulders before and after the main peaks emerged at concentrations too low to permit ultracentrifugal characterization. Ionization of the basic groups of the column was strongly affected by changes in pH between 7 and 8, and experiments not reported here showed that elution of RNP-I and RNP-II could be achieved at even lower salt concentrations if a slight rise in pH were incorporated into the salt gradient.

At the end of the NaCl–$MgCl_2$ gradient, 0.1 M Na_3PO_4 was pumped through the column and additional material having a high ratio of A_{260}:A_{280} (about 1.8) was eluted at 0.76 l. Although most of it emerged at pH values below 8, it is likely that all of it was exposed, at least briefly, to a much higher pH. Chemical analysis demonstrated the presence of both nucleic acid and protein in this peak, but efforts to characterize the material in the ultracentrifuge were unsuccessful because of its marked tendency to aggregate. Recovery of the material applied to the column was quantitative if the fraction eluted by 0.1 M Na_3PO_4 was included, and this was true of all the experiments described in this work.

tRNA from Particle-Free Fraction. That the more slowly

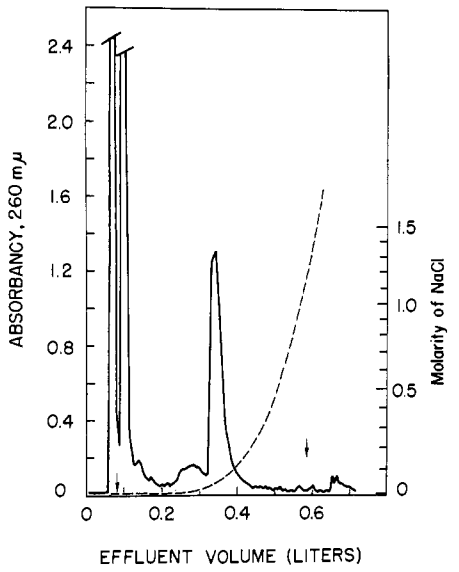

FIGURE 2: Elution profile resulting from the application of 20 ml of a particle-free preparation (total RNA = 2.32 mg). The sample was applied to the ECTHAM-cellulose column in two 10-ml portions, so the "pass-through" appears as two peaks (between 0.05 and 0.12 l.). No Triton X-100 was used. Conditions were otherwise the same as described in Figure 1.

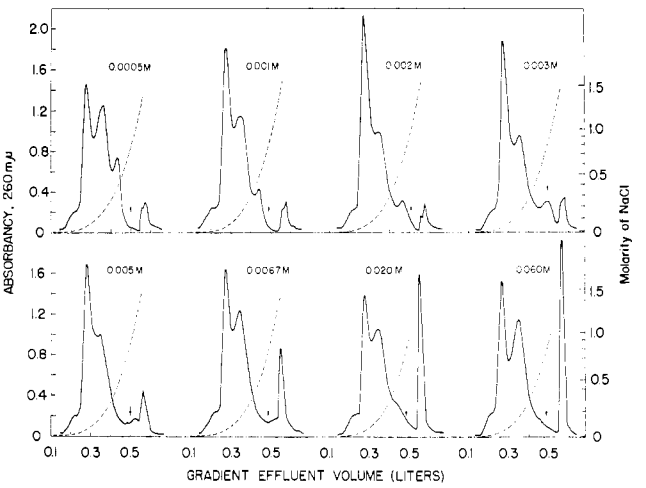

FIGURE 3: The effect of increasing Mg concentration in the salt gradient. Mitochondrial supernatant fraction applied and treated as in Figure 1, except that the $MgCl_2$ concentration in the limit buffer was at the value indicated above the corresponding pattern. The starting buffer contained 0.5 mM $MgCl_2$ in every case. "Pass-through" and Triton peaks are not shown. The broken line represents salt concentration measured and plotted as conductivity but translated into molarity of NaCl in scale at right. Abscissa scale indicates volume emerging after start of gradient.

sedimenting component in the first ribosome peak was tRNA was confirmed by applying to the column a clear, particle-free supernatant fraction obtained by centrifugation of a mitochondrial supernatant fraction at 105,000*g* for 90 min. Since the amount of tRNA to be expected in the usual sample volume was small, a double volume of the particle-free fraction was used in order to provide a peak of sufficient concentration to permit characterization in the ultracentrifuge. This was applied in two separate portions so that the usual column of Sephadex G-50 could be used for buffer exchange. After the first portion had entered the ECTHAM-cellulose column, the Sephadex column was disconnected, flushed with starting buffer to remove the low molecular weight components of the first sample, then reconnected for the application of the second portion. Consequently, the unadsorbed material emerged as a double peak, as shown in Figure 2. Since the sample contained no microsomes, treatment with Triton X-100 was omitted, and the gradient of NaCl was initiated soon after the sample had been washed into the ECTHAM-cellulose column with starting buffer. A major peak of material with an $A_{260}:A_{280}$ ratio of 1.95 emerged (at 0.34 l.) at the same position in the NaCl gradient as that normally occupied by RNP-I (0.04 M NaCl). Examination in the analytical ultracentrifuge showed it to be homogeneous, with a sedimentation coefficient of 3.8 S, an appropriate value for tRNA.

The tall tRNA peak was preceded by a low heterogeneous peak containing substances with $A_{260}:A_{280}$ ratios ranging from 0.75 to 1.1, similar in position and absorption ratios to the small shoulder observed in front of the first ribosome peak when a mitochondrial supernatant fraction was applied. Chemical determinations carried out on two fractions in this peak (at 0.26 and 0.28 l.) gave protein:RNA ratios of 290 and 87, respectively. On the other hand, the protein:RNA ratio for the tRNA peak was 1.3. Treatment with phenol resulted in the removal of two-thirds of the protein in the tRNA peak without changing the sedimentation coefficient or the chromatographic behavior of the RNA.

It should be noted in Figure 2 that very little remained to be eluted by the final 0.1 M Na_3PO_4 wash.

Ribosomes from Microsome Preparations. When microsome suspensions were applied to the ECTHAM-cellulose column and the standard procedure described above was carried out, the elution profiles were very similar to those obtained with mitochondrial supernatant fractions. As expected, however, the apparent size of the first nucleoprotein peak was usually diminished in microsome experiments by the absence of much of the tRNA that contaminated that peak in experiments employing mitochondrial supernatant fractions. Thus, in chromatograms from microsome preparations, the first ribosome peak (RNP-I) was generally smaller than the second (RNP-II).

Effect of Magnesium. The importance of Mg in maintaining the integrity of the ribosomal structure is well known, so a study of its effect on the chromatographic behavior of the ribosomal material was undertaken. When no Mg was added to the buffer used for chromatography, nearly all of the nucleoprotein emerged in the position of RNP-I, with a small shoulder at position II, and examination of material from the major peak in the ultracentrifuge revealed extensive dissociation of the ribosomes.

Figure 3 illustrates a series of experiments in which the Mg concentration was initially 0.5 mM in every case but rose at different rates in the course of the chromatogram. In effect, each succeeding pattern represents a higher Mg:Na ratio in the gradient portion of the chromatogram. At low values of this ratio an additional peak that will be referred to as RNP-III emerged immediately after RNP-II, at 0.43 l. It consisted of particles having sedimentation rates of about 44 and 26 S, but whether they had migrated on the column as preexisting particles or had arisen from a ribosome fraction, before or after emergence, as the result of dissociation in the rising NaCl

TABLE I: Effect of Increasing Sample Load of Microsomes in Successive Increments.

	Application[a]				
	1	2	3	4	Total
RNA-P adsorbed (μg)	500	380	188	118	1186
Lipid-P adsorbed (μg)	1167	811	172	63	2213
RNA-P/lipid-P					
Adsorbed	0.428	0.468	1.09	1.87	
Pass-through fraction	0.3	0.325	0.331	0.344	

[a] Each application contained 504 μg of RNA-P and 1180 μg of lipid-P, giving a ratio of 0.427.

concentration of the effluent, could not be determined. There was little if any nonparticulate material in this peak. Even at the lowest Mg:Na ratio used the first and second RNP peaks were similar in profile to those obtained under the standard conditions of chromatography. RNP-I was not examined in the ultracentrifuge. RNP-II, which was eluted at about 0.15 M NaCl, consisted primarily of 77S ribosomes; however, these particles underwent complete dissociation to subunits when the NaCl concentration was raised to 0.35 M, suggesting that the Mg ion was providing only marginal protection in this fraction. As the Mg:Na ratio was raised, RNP-III was shifted progressively to the right until it merged with the peak eluted by Na_3PO_4. At still higher Mg:Na ratios, additional ribonucleoprotein was shifted to enlarge that final peak. Presumably, aggregation was involved in this phenomenon.

Whereas the most satisfactory distribution of ribonucleoprotein peaks appeared to occur at the lower Mg:Na ratios, dissociation of the particles (as judged by their behavior in the analytical ultracentrifuge) was a serious problem. In the intermediate range, where both aggregation and dissociation were relatively low, the first and second ribosome peaks were less well resolved. Optimum conditions will evidently require the shape of the $MgCl_2$ gradient to be different from that of the NaCl gradient.

Load. The samples applied to the ECTHAM-cellulose columns were extremely heterogeneous. The microsomes, as fragments of the endoplasmic reticulum, represent a spectrum of particles varying widely in size and ribosome content, and there appeared to be a corresponding variation in their affinity for the column, inasmuch as the microsomes emerging in the "pass-through" fraction always exhibited a lower ratio of RNA to lipid-P than did the preparation that was applied. When the load was reduced by a factor of two, a larger proportion of the microsomes was bound, though the elution profile was not materially affected.

In one experiment the microsome sample was applied in four successive portions, each equivalent to half the standard load, and the column was washed with the starting buffer after each application. Even though the first application contained only half as much material as was used in previous experiments, not all of its microsomes were bound. The column was not saturated, however, for additional microsomes were adsorbed from the succeeding applications. Analysis of the four pass-through fractions permitted the calculation of the amounts of RNA and lipid phosphorus adsorbed at each application, and these values are shown in Table I. Nearly all of the RNA in the first application was adsorbed, 75% of that in the second, 37% of that in the third, and 24% of that in the fourth, totaling about 50% more RNA than in a standard experiment. The table also shows that the increments of adsorbed lipid phosphorus fell off more rapidly than those of the RNA. It is evident from the ratios of RNA-P to lipid-P that the material adsorbed in the later applications was progressively richer in RNA relative to lipid. This observation and the fact that the corresponding ratios for the pass-through fractions were appreciably lower and almost constant suggest that RNA-rich components in the later additions displaced from the column microsomes representing the low affinity end of the spectrum.

The nucleoprotein pattern obtained by elution was similar to that observed with a standard load of microsomes at the Mg level employed, except for an increase in the relative size of the later peaks. The peak eluted by Na_3PO_4 increased in quantity in rough proportion to the load, indicating that under these conditions it represented a distinct component in the applied sample, rather than an artifact arising from an excessive affinity of part of a component for certain sites on the column.

Protein Content. A small shoulder of material absorbing at 260 mμ invariably preceded the first RNP peak in the elution profile, even when microsome preparations largely freed of soluble protein were applied to the column. Absorbance at 280 mμ was nearly the same as at 260 mμ in this region and analysis by the Lowry–Folin method revealed that this shoulder contained protein comparable in magnitude to that in RNP-I and RNP-II, as shown in Figure 4. Because of the presence of this protein peak in close proximity to the ribosome peaks, the protein and RNA contents of the ribosomes were determined on pellets obtained by centrifuging these fractions. The ratio of protein to RNA was found to be 2.4 in ribosomes from the first peak and 1.9 in ribosomes from the second. The same result was obtained in two other experiments, indicating the validity of this small difference in protein content. Ribosomes obtained by conventional procedures employing deoxycholate were found to have a protein to RNA ratio of 1.1 when analyzed by the same procedure.

Rechromatography. Attempts to rechromatograph the ribosomes of RNP-I invariably resulted in their emergence at the position of RNP-II. This is illustrated in Figure 5, which shows that elution profile obtained when the ribosomes from a mitochrondrial supernatant fraction were eluted with a gradient having a limit Mg concentration of 2.5 mM. Since the ribosomes of the first peak were sedimented and resuspended in

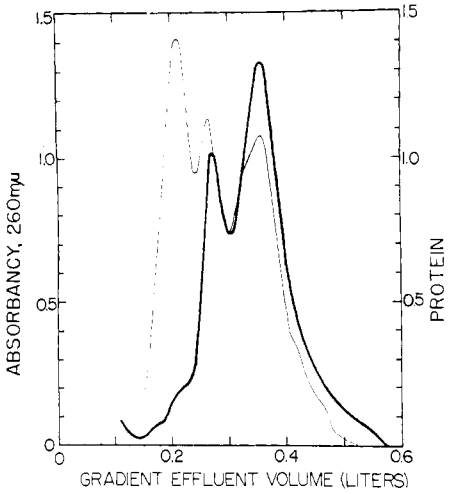

FIGURE 4: Protein content of "preribosome" peak. Sample applied as microsomes (total RNA = 10.6 mg), representing about 3 g of rat liver, treated with Triton X-100 and ribosomes eluted as in Figure 1, except that the terminal Na_3PO_4 elution was omitted. The heavy line represents absorbancy at 260 mμ. Thin line indicates protein content (in μg of protein/ml × 10^{-2}) by Lowry–Folin method, using bovine plasma albumin as a standard. The abscissa scale indicates volume emerging after start of gradient.

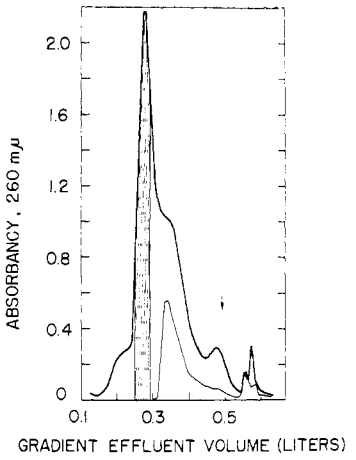

FIGURE 5: Rechromatography of ribosome peak I. Mitochondrial supernatant fraction corresponding to about 3 g of rat liver fractionated on ECTHAM-cellulose column as described in legend of Figure 1, except that limit solution of gradient apparatus contained 2.6 mM $MgCl_2$ instead of 20 mM. Heavy line represents absorbancy at 260 mμ. The shaded portion was pooled and centrifuged; the pellet was dispersed in starting buffer and rechromatographed under original conditions. The thin line shows absorbancy of rechromatographed material. The abscissa scale refers to volume emerging since start of gradient. The arrow indicates the point at which elution by 0.1 M Na_3PO_4 was begun.

fresh starting buffer before rechromatography, the tRNA was not present in the second application.

The same result was obtained when deoxycholate-treated ribosomes isolated by conventional centrifugation procedures were chromatographed under similar conditions, as is shown in the left section of Figure 6. The right-hand section of the figure demonstrates that storage of the ribosome preparation for 2 days at 0° prior to chromatography produced a partial transformation of RNP-I to RNP-II. The RNP-I peak in this case was prepared for rechromatography by diluting with three volumes of starting buffer (no centrifugation) with the result that whereas most of the material emerged at position II (0.38 l.), a small peak reappeared at position I (presumably nonsedimentable substance, at 0.32 l.). Similar results were encountered when the limit Mg concentration was 20 mM. The Mg concentration was not critical for this transformation, although the higher Mg level did increase the peak eluted by Na_3PO_4 after rechromatography.

The second RNP peak always emerged at its original position when rechromatographed, except for a variable amount (depending upon the Mg concentration) that appeared in the peak eluted by Na_3PO_4.

Plasma Cell Tumor. Microsomes prepared from RPC-20 plasma cell tumors produced ribosome elution profiles very similar to those obtained from rat liver microsomes, as is shown in Figure 7. As with liver, two ribosome peaks were eluted by the standard gradient and three at a lower Mg:Na ratio. However, all the ribosome peaks emerged significantly earlier in the gradient, evidently requiring lower salt concentrations for elution than the corresponding liver components.

The microsomes used in these two experiments were labeled *in vivo* with [^{3}H]uridine, and the incorporation of this label into RNA isolated from the fractions is represented by the broken lines in the figure. Comparison with the solid lines, representing absorbance of the RNA, reveals marked differences in the specific activity of the peaks. Peaks of higher specific activity are eluted later in the chromatogram, and that eluted by Na_3PO_4 had the highest specific activity of all. The heterogeneity of this peak is indicated by the fact that the number of counts emerging in this peak was diminished by only 29% whereas the amount of RNA fell by 76% when the lower Mg:Na ratio was used. Heterogeneity with respect to specific activity is also evident within the ribosome peaks eluted by the gradient.

The RNA emerging in the Triton X-100 eluate (at about 0.15 l.) contained very little radioactivity, whereas the pass-through peak showed a substantial number of total counts. The specific activity of the RNA in the latter fraction, however, was identical with that in the original unfractionated microsome preparation.

Recoveries of RNA and radioactivity were essentially quantitative. They were 97 and 94%, respectively, for the elution performed under standard conditions, and 99 and 98%, respectively, when the lower Mg:Na ratio was used.

Discussion

The chromatographic procedure described in this work offers a convenient and rapid means of obtaining clean, intact ribosomes from mammalian tissues such as rat liver and mouse plasma cell tumor. It is reasonable to expect it to be applicable to the isolation of bacterial ribosomes as well, although this has not yet been investigated. The ribosomes prepared from liver by this method contain significantly more protein, relative to RNA, than those obtained by conventional centrifugal methods employing sodium deoxycholate to disrupt the microsomal membrane. The reproducibility of the protein:RNA ratio suggests that the additional protein is part of a definite structure rather than an adventitious contaminant.

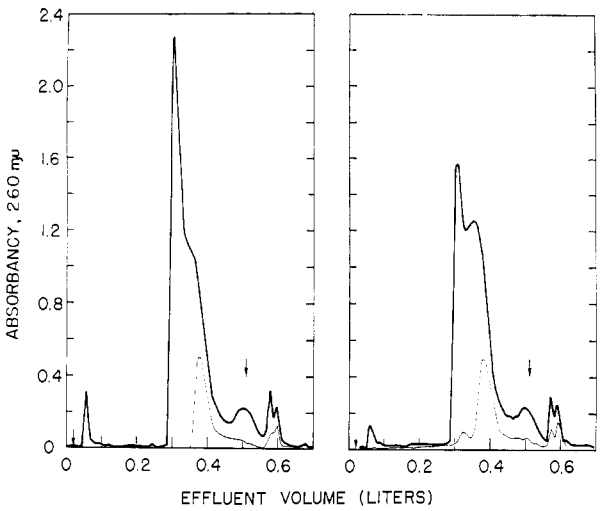

FIGURE 6: Chromatography of deoxycholate-treated ribosomes isolated from rat liver homogenates by conventional centrifugal procedures. Chromatographic conditions were the same as those of Figure 1, except that the limit solution of the gradient contained 3 mM $MgCl_2$ instead of 20 mM. Gradient was started at point indicated by first arrow; no Triton X-100 was used. Second arrow marks point at which elution with 0.1 M Na_3PO_4 was begun. Chromatogram at the left was obtained immediately after the centrifugal isolation of the ribosomes; that at the right was obtained with another portion of the same preparation after storage at 0° for 2 days. Both samples contained 5.5 mg of RNA. In each case the absorbancy of the effluent at 260 mμ is represented by the heavy line. The results of rechromatography of *ca.* 30-ml portions taken from the peaks at 0.3 l. are shown by the thin lines in each section. That at the left was prepared for rechromatography by centrifugation and resuspension in starting buffer; that at the right was simply diluted with three volumes of starting buffer.

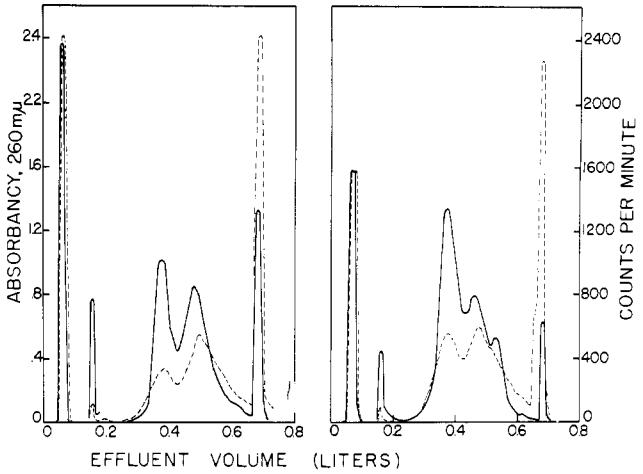

FIGURE 7: Chromatography of plasma cell tumor microsomes labeled with [^{3}H]uridine. In each experiment, 8 ml of the same microsome suspension (out of 17-ml total prepared from 4.7 g of tumor) was applied, providing 12.6 mg of RNA and 45,000 cpm. Elution conditions were the same as in Figure 1, except that in the experiment shown at the right the concentration of $MgCl_2$ in the limit solution of the gradient was 5 mM instead of 20 mM. The sample used in the right-hand section had been rapidly frozen and stored (−28°) for 13 days. The solid line represents the absorbance at 260 mμ of RNA isolated from the effluent fractions. The broken line indicates the radioactivity in the RNA of each fraction.

Experiments showing that these ribosomes are capable of synthesizing peptide chains in the presence of added mRNA are reported in another communication describing modifications of the buffers designed to provide directly the proper ionic milieu for amino acid incorporation, in particular, the substitution of potassium chloride for sodium chloride in the eluting gradient (Kedes *et al.*, 1969).

The appearance of two major peaks in the ribosome profile raises a question as to the nature of the difference between them, particularly since rechromatography invariably resulted in a shift of the first peak to the position occupied by the second. The fact that the column was nearly covered with microsomes initially might suggest that the first RNP peak was an artifact resulting from an overloading of the column. However, the material in RNP-I was obviously firmly adsorbed to the column, for it was not eluted until after treatment with Triton and after emergence of the protein-rich peak. Moreover, reduction of the sample size by a factor of two did not eliminate the first ribosome peak, as would have been the case if overloading had been involved. However, conversion as the result of an interaction with the column must be considered as a possibility.

The unusual situation in which the sample was initially adsorbed in the form of microsomes and then converted *in situ* to free ribosomes might also be suspected of contributing a special kind of heterogeneity to the conditions of adsorption, with the result that some of the ribosomes, being held less firmly than others, were released at a lower salt concentration. However, the double peak profile was observed even when ribosomes isolated from liver by the deoxycholate procedure (and presumably entirely free of membrane components) were chromatographed without the use of Triton. Similarly, plasma-cell tumor in which at least half of the ribosomes appear to be free of membranes (Kuff *et al.*, 1966), yielded chromatograms showing both peaks.

That RNP-I and RNP-II are distinct, though probably related, entities is indicated by their dissimilar sedimentation rates (83 and 77 S, respectively) and protein:RNA ratios (2.4 and 1.9, respectively). Larger scale experiments are contemplated with the view of isolating and studying the substances presumed to be split off during the conversion of the protein rich RNP-I to RNP-II. If the two peaks represent different functional states of the ribosome, such substances may include enzymic elements of the protein-synthesizing mechanism.

The small amount of protein eluted just before the ribosomes is similarly of interest. Since it was adsorbed to the column under conditions in which all the other soluble proteins of the liver homogenate were not, it is obviously unusual in some of its properties. This fraction is heterogeneous and apparently contains some nucleotidic substances, as evidenced by the fact that its absorbance at 260 mμ was approximately equal to that at 280 mμ. It is not unlikely that this material was released from the microsomes at the time of their disruption by Triton and was subsequently adsorbed to the column. Chromatography of particle-free supernatant fraction (Figure 2) showed the presence of similar material in the soluble portion of the homogenate, but this may have been the result of partial release when the endoplasmic reticulum was fragmented into microsomes during homogenization. Chromatograms obtained with microsome preparations, largely free of nonparticulate protein, invariably revealed the presence of this peak.

Larger scale experiments will be necessary to provide sufficient amounts from both particulate and nonparticulate sources to permit adequate comparison. A possible involvement of this fraction in protein synthesis should be considered in view of its association with structures known to be so involved.

A by-product of this work is the discovery that tRNA can be isolated from liver under very mild conditions by chromatography of a particle-free supernatant fraction on ECTHAM-cellulose. As obtained in these experiments, the tRNA fraction contained some protein, but whether this was a contaminant or was, at least in part, in combination with tRNA remains to be determined. The steeply curved salt gradient used in these experiments to achieve rapid elution of nucleoprotein having a wide range of elution requirements was not designed to provide a high degree of resolution in any particular portion of the chromatogram. It can be anticipated that the use of more suitable gradients will permit a wider separation of the protein prepeak from the tRNA with a consequent decrease in cross-contamination.

Reference

Bolton, E. T., Britten, R. J., and Cowie, D. B. (1959), *Carnegie Inst. Wash. Publ. 58*, 259.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Furano, A. V. (1966), *J. Biol. Chem. 241*, 2237.

Furano, A. V., and Hollis, V. W. (1967), *Fed. Proc. 26*, 612.

Kedes, L. H., Kuff, E. L., and Peterson, E. A. (1969), *Biochemistry 8*, 2923 (this issue; following paper).

Kuff, E. L., and Hymer, W. C. (1966), *Biochemistry 5*, 959.

Kuff, E. L., Hymer, W. C., Shelton, E., and Roberts, N. E. (1966), *J. Cell Biol. 29*, 63.

Kuff, E. L., and Zeigel, R. F. (1960), *J. Biophys. Biochem. Cytol. 7*, 465.

Littlefield, J. W., Keller, E. B., Gross, J., and Zamecnik, P. C. (1955), *J. Biol. Chem. 217*, 111.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Peterson, E. A., and Sober, H. A. (1959), *Anal. Chem. 31*, 857.

Peterson, E. A., and Sober, H. A. (1962), *Methods Enzymol. 5*, 3.

Salas, M., Smith, M. A., Stanley, W. M., Jr., Wahba, A. J., and Ochoa, S. (1965), *J. Biol. Chem. 240*, 3988.

Schneider, W. C., and Hogeboom, G. H. (1950), *J. Biol. Chem. 183*, 123.

Stanley, W. M., Jr., Salas, M., Wahba, A. J., and Ochoa, S. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 290.

Protein Synthetic Activity of Chromatographically Isolated Mammalian Ribosomes*

Laurence H. Kedes,† Edward L. Kuff, and Elbert A. Peterson

ABSTRACT: Ribosomes isolated from rat liver and rabbit reticulocytes by chromatography on ECTHAM-cellulose were active *in vitro* in an amino acid incorporating system. Microsome-containing fractions of rat liver, when subjected to the column procedure, yielded monosomes emerging in two peaks (RNP-I and -II), and these required the addition of synthetic polynucleotide for activity. RNP-I could function at a high level without the addition of "pH 5 enzymes" or soluble fraction. Chromatography of rabbit reticulocyte lysate yielded a single ribosomal peak (in the position of RNP-I) which contained active polysomes and, unlike the RNP-I obtained from rat liver, did not change its position on rechromatography. Purified pentameric ribosomes from reticulocytes were chromatographed without change in sedimentation rate or activity.

The accompanying paper (Peterson and Kuff, 1969) describes a method for the chromatographic isolation on ECTHAM-cellulose of intact 80 S ribosomes from mammalian tissues. The experiments reported here demonstrate the ability of ribosomes eluted with relatively low salt concentrations from this weak anion-exchange cellulose to support *in vitro* amino acid incorporation comparable to levels obtained with ribosomes prepared by conventional sedimentation techniques. Since K^+ is necessary to optimize the cell-free amino acid incorporation of animal ribonucleoprotein particles (Korner, 1961), gradient elution from ECTHAM-cellulose with KCl rather than NaCl was used in order to isolate RNP for direct utilization in *in vitro* systems. The chromatographic procedure provides a novel but practical method for the rapid isolation of large quantities of concentrated mammalian ribosomes which can be obtained free of membranes and can be used directly in amino acid incorporating systems.

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received January 30, 1969.*

† Present address: Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02114.