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.

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Protein Synthetic Activity of Chromatographically Isolated Mammalian Ribosomes*

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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.

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Experimental Procedures

Solutions. The following solutions were used: solution SSB, 0.25 M sucrose–5 mM Tris-HCl–1.5 mM $MgCl_2$; solution SB, 5 mM Tris-HCl–1.5 mM $MgCl_2$; solution A, 50 mM Tris-HCl–25 mM KCl–5 mM $MgCl_2$; solution RB, 50 mM Tris-HCl–50 mM KCl–1.5 mM $MgCl_2$; solution SBR, 5 mM Tris-HCl–0.1 M KCl–1.5 mM $MgCl_2$; and solution PB, 10 mM Tris-HCl–10 mM KCl–1.5 mM $MgCl_2$. The ratios of Tris to HCl in all solutions were 1.25:1, with a pH of 7.4, measured at room temperature. The molarity indicated for Tris hydrochloride is that of the chloride ion.

Preparation of Cell Fractions. Livers from male Buffalo rats were chilled, minced in solution SSB, and homogenized in glass tubes with a motor-driven Teflon pestle. Examination by phase microscopy ensured uniform cell breakage and minimal nuclear rupture. The 10,000*g* postmitochondrial supernatant fraction was used directly for chromatography, except where noted in the text or figures.

Reticulocytes from rabbits with phenylhydrazine-induced hemolytic anemia were generously supplied by Dr. M. Rabinovitz. The packed red cells were lysed by resuspension in two volumes of cold 1.5 mM $MgCl_2$ for 5 min and then diluted with four volumes of RB. The supernatant obtained from centrifuging this lysate for 15 min at 10,000*g* was used directly for chromatography or sucrose density gradient centrifugation.

The soluble fraction for [^{14}C]amino acid incorporation studies was prepared from liver homogenized in solution A. The mitochondrial supernatant fraction was then centrifuged at 105,000*g* for at least 3 hr, the lipid at the meniscus discarded, and the soluble fraction collected to within 3 cm of the microsomal pellet. A pH 5 fraction was prepared from the mitochondrial supernatant fraction by the method of Hoagland and Askonas (1963). The pellet was washed twice and resuspended in solution A. The final concentration of soluble fraction or pH 5 enzyme fraction added to a reaction mixture is reported as gram equivalents of tissue per milliliter.

Purified polysomes were prepared from rat liver by exposing a mitochondrial supernatant fraction to 1% deoxycholate (final concentration) and stacking 5 ml over a 4-ml layer of 0.5 M sucrose and a 3-ml layer of 1.5 M sucrose, each containing solution A (Wettstein *et al.*, 1963). After centrifugation at 105,000*g* for 4 hr, the pellet was washed and resuspended by gentle hand homogenization in solution PB and cleared by centrifugation at 10,000*g* for 10 min. Sucrose density gradient sedimentation was performed as described in the figure legends. Preparative zonal centrifugation of liver mitochondrial supernatant fraction or reticulocyte lysate was performed in a Spinco Model B-IV rotor (Kedes *et al.*, 1966).

Chromatography. Preparation, packing, equilibration, and regeneration of 10-g columns of the cellulosic ion-exchange adsorbent (ECTHAM-cellulose) is described in detail in the accompanying article (Peterson and Kuff, 1969). A 50-g preparative ECTHAM-cellulose column (45 × 2.5 cm) was packed with a roller pump. When the top of the column of adsorbent had settled under gravity, 15 psi of air pressure was applied with slight compression of the column to a final height of about 45 cm.

The eluting buffers in all the experiments with liver homogenate fractions were modified to contain KCl rather than NaCl. The starting buffer (SB) was 5 mM Tris-HCl–1.5 mM $MgCl_2$ (pH 7.4). Reticulocyte starting buffer (SBR) contained, in addition, 10 mM KCl. Gradient elution for 10-g columns was performed with a five-chamber Varigrad (Peterson and Sober, 1959), with starting buffer in the first four chambers and limit buffer in the fifth. Each chamber initially contained 100 g of solution. Limit buffer in all such experiments was 2.0 M KCl–5 mM Tris-HCl–3 mM $MgCl_2$ (pH 7.45).

After the applied sample had been washed in with starting buffer, the column was washed with a nonionic detergent, Triton X-100 (Z. D. Gilman Co., Washington, D. C.; 1 ml of a 0.9% solution per gram of adsorbent in the same buffer) in order to solubilize lipids, including microsomal membranes. Gradient elution did not yield peak ribosome concentrations of either RNP-I or RNP-II high enough to use these separated fractions directly in the cell-free system employed in this study. In order to obtain the necessary RNP concentrations preparative chromatography was performed by a single-step elution with 0.15 M KCl–5 mM Tris-HCl–1.5 mM $MgCl_2$ (pH 7.45). In several instances a two-step elution was performed in an attempt to reproduce the two populations of ribonucleoprotein particles that emerged with gradient elution (see Results). In these cases, 0.04 or 0.05 M KCl–5 mM Tris-HCl–1.5 mM $MgCl_2$ (pH 7.45) was used to elute the more lightly bound ribosomes.

Examination of Chromatographic Effluent. Material eluted from the ECTHAM-cellulose columns was examined for pH, conductivity, and absorbancy at 235, 260, and 280 mμ. Analytical ultracentrifugation was performed directly on peak fractions in a Spinco Model E centrifuge at 4–6° with an ultraviolet absorption system, and all values are reported as $s_{20,w}$. Photographic plates were scanned with a Joyce-Loebl microdensitometer. Sedimentation rates were calculated by a computer program, assuming a particle density $\rho = 1.56$. The RNA content of ribonucleoprotein preparations was determined as previously described (Peterson and Kuff, 1969).

Cell-Free Incorporation Techniques. Liver ribonucleoprotein particles eluted from ECTHAM-cellulose or sedimented from cell sap were incubated at 37° in a reaction mixture containing, in 1 ml: ATP, 1 μmole; GTP, 0.5 μmole; phosphoenolpyruvate, 5 μmoles; phosphoenolpyruvate kinase, 40 μg; [^{12}C]amino acids (omitting phenylalanine), 0.04 μmole each; [^{14}C]phenylalanine, 1.43 mμmoles (specific activity 140 μCi/μmole; Nuclear-Chicago, Inc.); [^{12}C]phenylalanine, 8 mμmoles; β-mercaptoethanol, 6 μmoles; $MgCl_2$, 4.4 μmoles; KCl, 50 μmoles; and Tris-HCl, 60 μmoles (pH 7.45). When RNP particle fractions were compared in their ability to incorporate amino acids into proteins, the final electrolyte and buffer content of the reaction mixtures was made identical by taking into account differences in their initial electrolyte composition. When soluble fraction rather than pH 5 fraction was used as a source of transfer RNA and enzymes, however, its contributions of cell sap, electrolytes, amino acids, and nucleotides were in addition to the listed components.

Reactions were carried out in prewarmed conical centrifuge tubes. Soluble or pH 5 enzyme fraction and ribosomes were added in rapid sequence. The incubation at 37° was stopped by the addition of an equal volume of cold 10% trichloroacetic acid containing 20 mM phenylalanine. An appropriate aliquot of soluble or pH 5 fraction was added to equalize the precipitate in each mixture. The precipitates were washed twice with the trichloroacetic acid–phenylalanine solution, heated to 80° for 30 min, washed with cold trichloroacetic

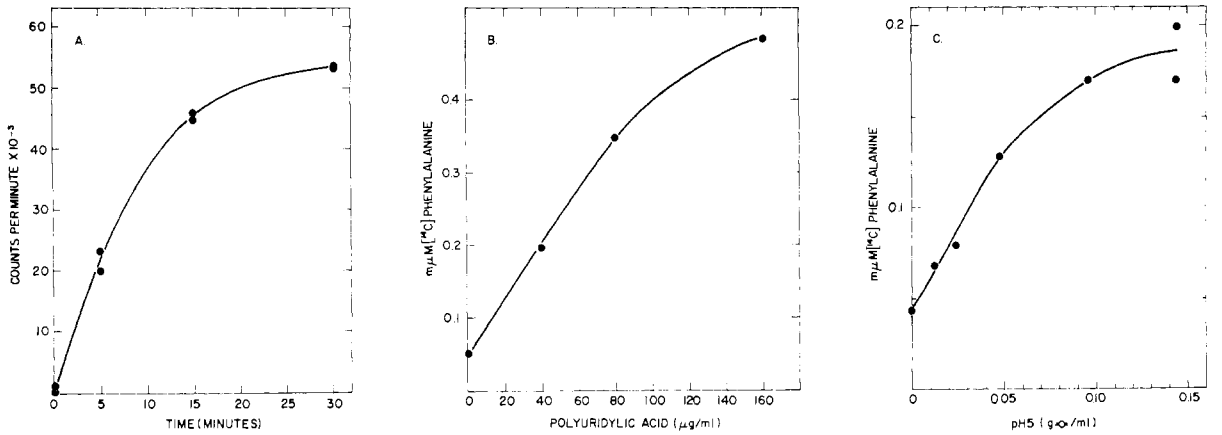

FIGURE 1: Characteristics of the *in vitro* amino acid incorporating activity of rat liver ribosomes (RNP-I + RNP-II) isolated by single-step elution from ECTHAM-cellulose. Details of the incorporation system are described in Experimental Procedures. The incorporation of [^{14}C]phenylalanine into protein is expressed as either counts per minute (A) or millimicromoles (B and C) per milligram of RNA added in the RNP fraction (RNP-RNA). (A) Time course of incorporation of [^{14}C]phenylalanine into protein in the presence of poly U (100 μg/ml) and liver-soluble fraction (0.0167 g-equiv/ml). Reaction mixtures contained 0.11 mg of RNP-RNA. (B) Response of phenylalanine incorporation to poly U concentration in reaction mixtures containing 0.096 g-equiv of rat liver pH 5 fraction and 0.15 mg of RNP-RNA. (C) Response to pH 5 fraction in mixtures containing 80 μg of poly U/ml and 0.19 mg of RNP-RNA.

acid, and transferred quantitatively to filter paper disks. Each disk was rinsed on a Millipore filtration apparatus, thrice with ethanol–ether (3:1, v/v) and thrice with methanol. It was then wetted with toluene and placed in a scintillation vial with 4 ml of toluene containing 4 g of 2,5-diphenyloxazole and 0.5 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per l. Samples were counted in a Packard TriCarb liquid scintillation counter at 50% efficiency for ^{14}C. In several experiments the reaction was stopped by adding chilled 20 mM phenylalanine and icing the reaction tube. The mixtures were then transferred to filter paper cylinders, precipitated with chilled 10% trichloroacetic acid, and washed by a modification (Kuff *et al.*, 1966) of the method of Mans and Novelli (1961).

Nascent protein from reticulocytes that had been incubated with [^{14}C]leucine was precipitated from sucrose density gradient or chromatographic fractions by an equal volume of cold 10% trichloroacetic acid containing 20 mM [^{12}C]leucine. Bovine serum albumin (100 μg, Armour and Co.) was added as carrier. After being heated at 90° for 10 min, the precipitate was layered onto Millipore filters and washed with cold 5% trichloroacetic acid and chloroform–ether–ethanol (1:2:2, v/v). Dried filters were placed in vials with scintillation fluid and counted as described above.

RNP prepared from reticulocytes by either chromatography or sedimentation was incubated with reticulocyte soluble fraction in the reaction mixture previously described except that [^{14}C]leucine was used in place of [^{14}C]phenylalanine. Trichloroacetic acid precipitates were resuspended in ether–petroleum ether (1:3, v/v), dried evenly on planchets, and counted in an end-window gas-flow analyzer (Nuclear-Chicago Co.), with corrections for self-absorption.

Results

Comparison of KCl and NaCl in the Chromatography of Liver Ribosomes. A number of preliminary experiments were carried out with rat liver microsome and mitochondrial supernatant fractions to ensure the identity of the RNP chromatographic peaks when (a) homogenates were prepared in solution SSB rather than in 0.25 M sucrose alone, and (b) KCl was used as the eluting salt instead of NaCl (*cf.* Peterson and Kuff, 1969). The molar ratio of KCl to $MgCl_2$ required to produce a similar chromatogram was determined, and the $MgCl_2$ concentration in the limit buffer was decreased, accordingly, to 3 mM. This concentration of Mg ion is known to preserve mammalian RNP particles (Warner *et al.*, 1963). The absorbancy ratio (260:280 mμ) of the RNP peaks obtained by elution with KCl was the same (about 1.8) as that reported for the corresponding peaks obtained by elution with NaCl. Material from KCl-eluted RNP-I consisted primarily of 83S particles, accompanied by a small amount of 3.8S RNA and traces of a few heavier components, whereas RNP-II contained only 77S

TABLE 1: Characteristics of Amino Acid Incorporation with Chromatographically Prepared Liver Ribosomes.

Reaction Mixture	Poly U (μg/ml)	cpm[a]
Complete[b]	160	1949
	80	1274
	40	984
	0	174
Minus soluble fraction	160	939
	0	48
Minus ribosomes	160	0

[a] Counts per minute of [^{14}C]phenylalanine incorporated into hot trichloroacetic acid insoluble protein. [b] The complete mixtures contained 0.12 mg of RNA of liver ribosomes (RNP-I + RNP-II) prepared by single-step elution (see text), soluble fraction equivalent to 0.01 g of liver, and poly U at the indicated final concentrations. Other components are specified under Experimental Procedures.

TABLE II: Ability of Chromatographically Prepared Mammalian Ribosome Fractions to Support Amino Acid Incorporation in the Presence of Added Enzyme Fractions.

Expt.	Ribosome Source	Poly U (μg/ml)	Soluble Fraction[a]			pH 5 Fraction[b]		
			0	Max	0/Max	0	Max	0/Max
Rat Liver								
1[c]	Polysomes	0	965	11,450	0.08			
	RNP-I + RNP-II[d]	100	1,655	7,075	0.23			
2	RNP-I + RNP-II[d]	100				0.043	0.185	0.23
3	RNP-I	160	7,825	16,240	0.48			
	RNP-II	160	1,280	7,366	0.17			
4	RNP-I	160				0.499	0.617	0.81
	RNP-II	160				0.076	0.738	0.10
Rabbit Reticulocytes								
5[e]	Polysomes	0	21,700	67,600	0.32			
	RNP-I	0	3,610	55,500	0.065			

[a] Counts per minute of ^{14}C-labeled phenylalanine (expt 1 and 3) or leucine (expt 5) incorporated into hot trichloroacetic acid insoluble protein per milligram of RNA added in ribosome fractions. 0, incorporation with no added soluble fraction; Max, maximum incorporation with added soluble fraction. [b] Values expressed as mμmoles of [^{14}C]phenylalanine incorporated into protein per milligram of RNA added in ribosome fractions. [c] Separate aliquots of a mitochondrial supernatant fraction were used for parallel preparation of the polysome fraction by sodium deoxycholate treatment (see Experimental Procedures) and the RNP fraction by chromatography. [d] Single-step elution. [e] Separate aliquots of a clarified lysate were used for preparation of the polysome fraction (centrifugation of the lysate at 105,000*g* for 90 min, resuspension of the pellet in PB) and RNP-I fraction (chromatography).

particles. These sedimentation coefficients were the same as those obtained by elution with NaCl.

Amino Acid Incorporating Activity of Chromatographically Isolated Liver RNP Particles. SINGLE-STEP ELUTION. Preparative RNP chromatography was carried out on a 50-g ECTHAM-cellulose column to which was usually applied a mitochondrial supernatant fraction equivalent to 30 g of liver, with the pH adjusted to 7.4. The column was subsequently washed with starting buffer, then with 0.9% Triton X-100, and elution was carried out in one step as described under Experimental Procedures. Peak fractions emerged with absorbancies (260 mμ) as high as 30. Aliquots were used directly for amino acid incorporation studies.

The general incorporation characteristics of the RNP fraction isolated by single-step elution (RNP-I plus RNP-II) are shown in Tables I and II and Figure 1. Incorporation of phenylalanine into peptide insoluble in hot trichloroacetic acid proceeded most rapidly in the first 10 min but was continuous throughout the 30-min incubation (Figure 1A). The system was strongly dependent upon the addition of exogenous mRNA in the form of poly U (Figure 1B and Table I); with 160 μg/ml of poly U in the reaction mixtures (highest level tested), the incorporation of phenylalanine was generally 10–12 times the level observed in the absence of poly U. The requirement for added soluble enzymes was less strict, however (Figure 1C, Tables I and II, expt 1 and 2). At comparable concentrations of poly U, the incorporation in the absence of either pH 5 or soluble fraction ranged from 23 to 45% of that observed at the maximum stimulatory concentration of the enzyme fraction.

Centrifugally purified liver (poly)ribosome fractions differed from the chromatographically isolated RNP preparations in showing a greater dependence upon added soluble enzyme (Table II) and an essentially complete lack of response to added poly U (Figure 2).

DOUBLE-STEP ELUTION. When liver ribosomes were eluted in two steps as described in Experimental Procedures, the peak fractions showed differences in their sedimentation rates corresponding to those shown by RNP-I and RNP-II when eluted by a continuous gradient. The relative ability of ribosomes from each of the two peaks to support amino acid incorporation varied somewhat from experiment to experiment (Table II). No reproducible differences were observed when the fractions were tested in the presence of added pH 5 or soluble fractions. However, RNP-II, eluted at the higher KCl concentration, consistently showed a much stricter requirement for added enzyme fractions than did RNP-I (Table II).

In general, both the low endogenous activity (in the absence of poly U) and the predominantly monomeric form of chromatographically isolated liver ribosomes suggested a loss or degradation of mRNA during the preparative procedure (see Discussion). Attention was therefore turned to a comparative study of rabbit reticulocyte polyribosomes as a more defined system in which to evaluate the effects of chromatography.

Chromatography of Reticulocyte Ribosomes. Clarified reticulocyte lysates in RSB were applied directly to an ECTHAM-cellulose column. Both KCl and NaCl gradients eluted only one peak, which emerged in the RNP-I position, and very little material was eluted by 0.1 M Na_3PO_4 (Figures 3 and 4). This RNP-I had an absorbancy ratio (260:280 mμ) of 1.80, but unlike the RNP-I fraction of rat liver or myeloma tumor (Peterson and Kuff, 1969), it was stable on rechromatography and was not converted into more tightly bound material.

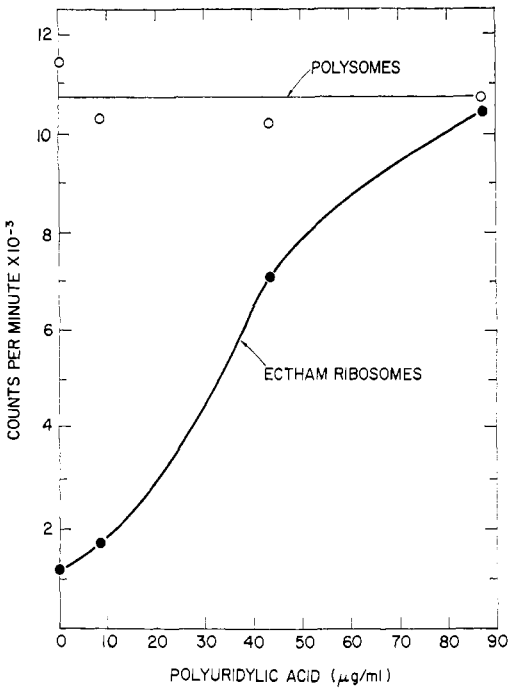

FIGURE 2: Response of chromatographically prepared liver RNP (RNP-I + RNP-II, single-step elution) to increments of poly U compared with lack of response of centrifugally purified liver polysomes in the *in vitro* amino acid incorporating system. Incorporation is expressed as counts per minute in protein/mg of RNA added in RNP fraction. The reaction mixtures contained, in addition to the components detailed under Experimental Procedures, 0.087 g-equiv of liver soluble fraction and RNP or polysomes representing 0.4 mg of RNA.

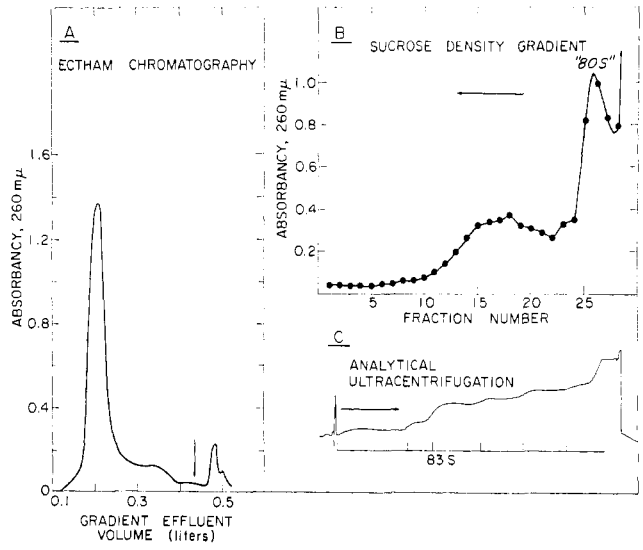

FIGURE 3: Elution patterns and sucrose density studies. (A) Elution pattern obtained when the lysate from 1.5 ml of packed rabbit reticulocytes, diluted to 10 ml with SBR, was applied to a 10-g column of ECTHAM-cellulose equilibrated with the same buffer. The column was washed with 16.5 ml of 0.9% Triton X-100 in SBR, and a gradient to 2.0 M KCl–5 mM Tris-HCl–2 mM $MgCl_2$ (pH 7.45) was started. Only the gradient and Na_3PO_4 (introduced at arrow) portions of the chromatogram are shown. (B) Sucrose density gradient analysis of another aliquot of the same lysate: 1 ml was layered over a 28-ml gradient of 15–30% sucrose containing SBR and centrifuged at 25,000 rpm in the SW25.1 rotor of a Spinco Model L ultracentrifuge for 80 min. The tube was punctured, fractions were collected volumetrically, and their absorbancies were measured at 260 mμ. Direction of sedimentation is indicated by the arrow. The peak corresponding to the monomeric ribosomes (Warner *et al.*, 1963) is designated 80S; the various size species of polysomes lying deeper in the tube (fractions 10–20) were not resolved in this run (*cf.* Figure 4A). (C) An aliquot of the pooled chromatographic fractions composing the main absorbancy peak in section A was subjected to analytical ultracentrifugation in a Spinco Model E centrifuge with ultraviolet optics (see Experimental Procedures). The thin line represents a densitometric tracing of the photographic negative exposed 13 min after the rotor reached a speed of 25,980 rpm at 3.5°. Arrow shows direction of sedimentation. At least five discrete boundaries are evident as indicated along abscissa; two additional components had sedimented to the bottom of the cell by the time of the exposure. The $s_{20,w}$ of the main component was computed to be 83 S.

Ultracentrifugal analysis of the RNP in the major peak revealed a spectrum of sedimentation rates consistent with the presence of polysomes (Figure 3).

The stability of the polysomes was further investigated by isolating specific polysome sedimentation classes from a reticulocyte lysate by centrifugation through a 15–30% sucrose gradient in solution SBR in a Spinco B-IV rotor. Fractions composing the monomer and pentamer peaks were diluted with large volumes of SB to reduce the viscosity and then applied to identical 10-g columns of ECTHAM-cellulose. In each case, a single sharp RNP peak was eluted in the position of RNP-I with a KCl gradient. Recovery of optical density was greater than 95%. The absorbancy ratio (260:280 mμ) was 1.80 in both cases. When examined in the ultracentrifuge, the eluted fractions presented homogeneous sedimentation boundaries, with $s_{20,w}$ values of 80 and 237 for the monomer and pentamer peaks, respectively. There was no evidence of lighter or heavier particles in either peak, indicating that the pentameric reticulocyte polysomes were stable through chromatography on ECTHAM-cellulose.

Reticulocytes were incubated at 37° with [^{14}C]leucine for 2 min and then lysed. An aliquot of clarified lysate was analyzed in a sucrose gradient (Figure 4A) to confirm the localization of nascent peptides on polysomes (Warner *et al.*, 1963). Another aliquot was chromatographed on ECTHAM-cellulose as illustrated in Figure 4B. A major peak of labeled protein emerged with the RNP particles during chromatography. This labeled protein sedimented with the ribosomes at 105,000-*g*, indicating that the radioactive material was not a special class of soluble proteins that happened to be eluted in the RNP position.

Amino Acid Incorporating Activity of Chromatographically Isolated Reticulocyte Polysomes. Reticulocyte polysomes are known to contain endogenous message and to function *in vitro* in the synthesis of hemoglobin chains (Allen and Schweet, 1962). As shown in Table II (expt 5), a preparation of reticulocyte polysomes prepared by differential centrifugation was highly active in incorporating [^{14}C]leucine into hot trichloroacetic acid insoluble protein in the absence of added mRNA. Chromatographically prepared reticulocyte polysomes (RNP-I, single step elution) were almost equally active, indicating little loss of endogenous message during chromatography. In contrast to the experience with liver, reticulocyte polysomes eluted from ECTHAM-cellulose showed a strong requirement for soluble fraction.

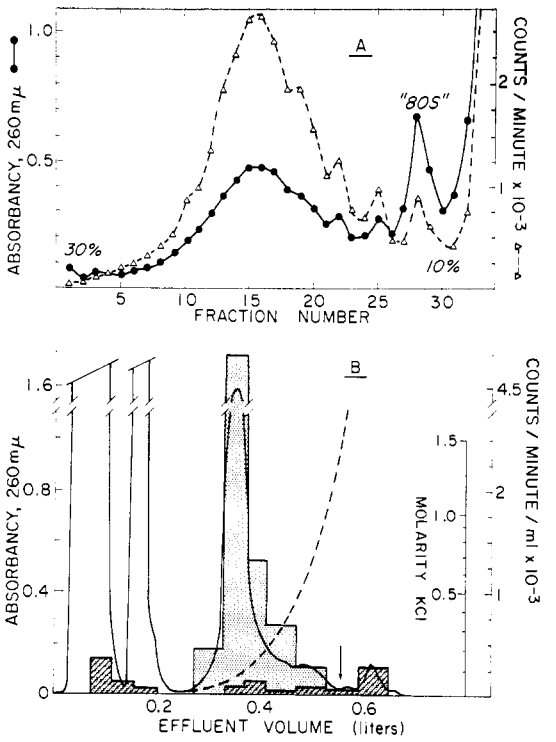

FIGURE 4: Nascent peptide studies. (A) Sucrose density gradient analysis of an aliquot of lysate prepared from rabbit reticulocytes that had been incubated with [^{14}C]leucine for 2 min. The lysate was layered over a 30-ml gradient of 10–30% sucrose containing SB and centrifuged for 150 min at 25,000 rpm in the Spinco SW25.1 rotor. Fractions were collected and analyzed for absorbancy as in Figure 3B. Protein radioactivity was assayed as described in Experimental Procedures. (B) An aliquot of the same lysate was applied to a 10-g ECTHAM-cellulose column and eluted as in Figure 3A. Fractions were pooled and examined for protein radioactivity (shaded areas). Aliquots of the pooled fractions were also centrifuged at 105,000*g* for 2 hr (Spinco no. 40 rotor) and the nonsedimentable supernatant material in each was examined for protein radioactivity (striped areas).

Discussion

These experiments demonstrate that mammalian ribosomes prepared by ECTHAM-cellulose chromatography are able to support the incorporation of amino acids in a cell-free system. Low salt concentrations (less than 0.2 M) sufficed to elute active 80S particles from the ECTHAM-cellulose, thus providing a novel and potentially useful system for the study of mammalian ribosome function.

Liver ribosomes prepared by single-step elution (RNP-I plus RNP-II) showed only partial dependence on exogenously supplied enzyme and tRNA fractions. When the two RNP populations were separated (two-step elution), it was clear that the requirement of RNP-I for additional enzymes was less rigorous than that of RNP-II. This functional difference indicates that the relatively high protein content of RNP-I ribosomes (Peterson and Kuff, 1969) reflects, at least in part, a larger complement of the enzymes necessary for protein synthesis.[1] The ready conversion of RNP-I to RNP-II (lower protein content, stricter dependence on added factors) further suggests that such enzymes are loosely bound to the RNP-I ribosomes. In this event, the RNP-I fraction of liver could be a useful source of factors which in conventional isolation techniques are removed from the ribosomes and must be recovered from the soluble phase.

On the other hand, our experience with the reticulocyte system reveals that enzyme complement cannot be regarded as the sole factor determining the elution behavior of various types of ribosomes on ECTHAM-cellulose. Reticulocyte ribosomes were consistently eluted in the RNP-I position (and failed to convert on rechromatography) although they exhibited a much stricter requirement for added soluble fraction in the amino acid incorporating system than did the RNP-I ribosome fraction from liver.

It is well known that the ribosomes of normal liver occur chiefly in polysomal form (and predominantly bound to microsomal membranes). Nevertheless, ribosomes prepared from liver by ECTHAM-cellulose chromatography appeared almost exclusively as monomers. The reticulocyte studies (in which polysomes were added free of membranes) demonstrated that the various steps involved in chromatography (binding to the column, treatment with Triton X-100, elution) were not intrinsically destructive of polysomes. It is possible, in the case of liver, that solubilization of membranes by the Triton liberated nucleases that destroyed RNA strands bridging the polysomes. Alternatively, the presentation of polysomes in a membrane-bound (extended ?) form to the ion exchanger may have facilitated a tight binding of mRNA and subsequent elution of messenger-free monomeric ribosomes. At any rate, the fact that the eluted liver ribosomes responded promptly and strongly to the addition of poly U in the incorporation mixture suggests that they had been stripped of native messenger at some stage in the chromatographic procedure. Thus they provide an active system in which to test the ability of artificial or naturally prepared message material to stimulate protein synthesis.

The above considerations prompted us to chromatograph centrifugally purified liver polysomes prepared by deoxycholate treatment as described under Experimental Procedures. The elution pattern did not differ significantly from that obtained with whole liver microsomes, and RNP-I was again found to convert into a more tightly bound state upon rechromatography. Analytical ultracentrifugation of the RNP-I fraction revealed a discrete 80S component representing about one-third of the total ultraviolet-absorbing material, the remainder consisting of more rapidly sedimenting but poorly resolved components which may have been polysomes. The RNP-II fraction consisted primarily of monomers, with a trace of ribosomal subunits.

It appears, therefore, that neither the physical state of the applied ribosomes (*i.e.*, free *vs.* membrane bound) or their complement of bound enzyme protein can be strictly correlated with their chromatographic behavior on ECTHAM-cellulose under the conditions employed. Whether the elution properties of the ribosomes reflect other surface properties or configurational states remains to be determined.

[1] In addition, 4S RNA, presumably containing tRNA, was eluted from ECTHAM-cellulose in the RNP-I position, and may have contributed to the relative independence of RNP-I fractions with respect to added soluble factors in the *in vitro* amino acid incorporation.

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Some Environmental Effects on the Thermal Transition of *Bacillus amyloliquefaciens* Ribonuclease (Barnase)*

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ABSTRACT: The thermal transition temperature, T_m, of barnase is unaffected by pH in the range 5–9, but decreases outside of this range.

Below pH 5 the T_m closely parallels that of pancreatic ribonuclease and, as with that enzyme, the decrease in T_m at low pH is sharply reduced by sulfate. The T_m is not markedly affected by phosphate at any pH. The increase in proton binding which accompanies unfolding has a maximum of 3 or 4 at about pH 3.5, suggesting a similar number of abnormal carboxyl groups. Only two of the six tyrosines are ionized at pH 12.2 in the folded form. Above pH 7, stability is reduced by 10^{-6} M Cu^{2+}; 10^{-5} M guanosine monophosphate has little effect on the T_m but reduces the solubility of the unfolded form.

The general nature of the thermal transition of barnase has been documented in a previous paper (Hartley, 1968). It was shown that the reaction could be considered, to good approximation, to progress as a single step from the native folded form to a completely disorganized random coil. The reaction is reversible and the two forms are in equilibrium with one another in the transition range. Since barnase has no covalent cross-links (Lees and Hartley, 1966), it follows that the amino acid sequence fully determines the folded structure of the active enzyme. Studies of this transition in various environments and with various chemical and genetic modifications of the enzyme should yield clues as to how the different residues contribute to the form and stability of the folded molecule.

In this paper are reported the effects of various reagents on the transition of the unmodified, wild-type enzyme, with particular reference to the hydrogen ion concentration.

Materials and Methods

Our procedure for the production and purification of barnase has undergone more or less continuous modification since last reported (Lees and Hartley, 1966) and an up-to-date description is in preparation. The product, however, is similar, perhaps slightly purer, and certainly more reliably free of contaminating proteases. The last point is of utmost importance for the type of experiments reported here (and in Hartley, 1968). The enzyme in its folded form is quite stable in the presence of such proteases, but as the transition temperature is approached (in a variety of solvents) the unfolding reaction is rapidly driven to completion by the digestion of the unfolded form.

Methods for obtaining spectra and for spectrophotometric and gel filtration observations of the transition, etc., were reported previously (Hartley, 1968).

The pH values reported were measured with a Corning Model 12 pH meter. Those of the solutions used in the spectrophotometric transition experiments were determined directly after the experiments.

Results

Figure 1 shows the pH dependence of the transition temperature below pH 8. These data were all determined with the ionic strength ($\Gamma/2$) at 0.1, except at pH 0.7 (0.2 N HCl), and the two points for which NH_4Cl was added to make $\Gamma/2 = 0.2$. The points marked by open circles represent runs in which no buffer was included, the salt being NH_4Cl. For the other points, various buffers were used as noted in the figure legend. That some care must be taken in the choice of buffers may be seen. The transition temperature was depressed by the excess of acetic acid below its pK and, similarly, by basic imidazole above its pK. The same effect occurs with Tris in the upper end of its buffering range, but formic acid appears to be innocuous.

Below pH 0.7, reliable transition temperatures are difficult to obtain due to the decrease in solubility of the unfolded

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