The Effect of High Ionic Strength on the Sedimentation Properties of the Biologically Active Component of Bacterial Ribonucleic Acid

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1. The sedimentation properties of the fraction of bacterial RNA which stimulates the incorporation of amino acids into acid-insoluble material *in vitro* depend on the ionic strength of the sedimentation medium. 2. The different distributions of stimulatory activity found in centrifuged linear sucrose gradients loaded with bacterial RNA and containing 0.1M- or 0.6M-sodium chloride resemble closely the different sedimentation profiles of rapidly labelled RNA observed under the same conditions. 3. These findings agree with those of previous work of a similar nature (Willson & Gros, 1964) and demonstrate that the component of bacterial RNA preparations which stimulates the incorporation of amino acids into acid-insoluble peptides *in vitro* is contained in their rapidly labelled fraction.

Since the introduction of the 'messenger-RNA hypothesis' (Jacob & Monod, 1961) and the discovery of a rapidly turning over fraction of bacterial RNA different from ribosomal and soluble RNA (Gros *et al.* 1961b) evidence has accumulated that the messenger RNA is contained in this rapidly turning over RNA fraction. Thus it has been observed that a part of this RNA can form double-stranded hybrids with one strand of its homologous DNA (Bolton & McCarthy, 1962, 1964) and that this hybridizable fraction of rapidly labelled RNA has a base composition resembling closely that of its homologous DNA (Monod, Jacob & Gros, 1962; Midgley & McCarthy, 1962).

Other lines of evidence also point to a close relationship between rapidly labelled RNA and messenger RNA. Rapidly labelled RNA forms reversible complexes with 70S ribosomes (Tissières, Schlessinger & Gros, 1960; Gros *et al.* 1961a) and these complexes are the site of protein synthesis (Marks, Burka & Schlessinger, 1962; Gilbert, 1963; Gierer, 1963; Warner, Knopf & Rich, 1963). Further evidence of a different nature was obtained in studies of the synthesis and the degradation of rapidly labelled RNA which occur under certain defined conditions. Synthesis of rapidly labelled RNA accompanies induction of new enzymes in bacterial cultures (Attardi, Naono, Rouvière, Jacob & Gros, 1963; Hayashi, Spiegelman, Franklin & Luria, 1963) and degradation of this

RNA is observed in bacteria treated with actinomycin D (Levinthal, Keynan & Higa, 1962), or with 2,4-dinitrophenol or proflavine (Soffer & Gros, 1964). These situations are characterized by a parallel appearance and disappearance of rapidly labelled RNA; of RNA capable of forming a hybrid with homologous DNA; and of protein-synthesizing activity, which strongly supports the view that at least part of the rapidly labelled RNA functions as a template for protein synthesis.

Finally a direct demonstration of the role of at least a part of rapidly labelled RNA as template for protein synthesis was obtained in studies which showed that the sedimentation characteristics of the component of bacterial RNA preparations which stimulates amino acid incorporation *in vitro* and of their rapidly labelled fraction were identical (Nirenberg & Matthaei, 1961; Tissières & Hopkins, 1961; Willson & Gros, 1964).

It has been shown (Asano, 1963; Hayes, Guérin & Hayes, 1963; Hayes, Hayes & Guérin, 1966) that the sedimentation properties of rapidly labelled RNA depend on the ionic strength of the sedimentation medium. The present paper shows that the rapidly labelled component of bacterial RNA and the component which stimulates amino acid incorporation *in vitro* possess identical sedimentation properties in both high and low ionic strength media. Thus a change in one parameter of the sedimentation system modifies greatly, and in identical fashion, the behaviour of rapidly labelled RNA and of the RNA fraction which stimulates

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amino acid incorporation *in vitro*. A similar finding made with bacteriophage T₄ messenger RNA has been reported (Bautz, 1963).

MATERIALS AND METHODS

Chemicals. Reagent-grade sucrose for use in density-gradient centrifugations and in the preparation of the incorporation system *in vitro* was obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex. Radioactive ¹⁴C-labelled amino acids were products of The Radiochemical Centre, Amersham, Bucks. and of the New England Nuclear Corp., Boston, Mass., U.S.A. Potassium polyvinyl sulphate and Actinomycin D were supplied by the Eastman Organic Chemical Co., Rochester, N.Y., U.S.A., and ribonuclease and deoxyribonuclease by the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Organisms. *Escherichia coli* strains B, ML 308 and 200 PS Flac⁺ were obtained from Professor F. Jacob, Institut Pasteur, Paris 15, France.

Growth of bacteria. Cells used for the preparation of 'S 30' extracts and unlabelled RNA were grown in mineral medium 63 (Monod, Cohen-Bazire, & Cohn, 1951) supplemented with vitamin B₁ (100 µg./l.) and glucose (4 g./l.) and harvested in the exponential phase.

Pulse-labelled RNA preparations were obtained by using cultures grown on a tris-buffered medium containing tris, pH 7.5 (0.1 M), NH₄Cl (0.02 M), MgSO₄ (2 mM), FeCl₃ (10 µM), K₂HPO₄ (100 µM), glycerol (2 g./l.) and vitamin B₁ (100 µg./l.).

Pulse labelling. Cultures were grown in the low phosphate medium described above with vigorous agitation at 37°. Generation times were between 1½ and 2 hr. and growth stopped at *E*_{420 mμ} approx. 1 from the phosphate exhaustion. At *E*_{420 mμ} 0.8, carrier-free [³²P]orthophosphate (0.1 mc/ml. of culture) was added for 25–35 sec. (0.5% of the generation time). At the end of the labelling period the cultures were poured rapidly on to sufficient crushed ice and 1.0 M-Na₂S₂O₃ solution to produce in the final mixture a temperature of 0° and a Na₂S₂O₃ concentration 0.01 M. Cells were harvested by centrifugation and washed once with tris-HCl buffer, pH 7.4 (5 mM), containing magnesium acetate (0.01 M), and either extracted immediately or stored frozen at -15°.

Preparation and fractionation of bacterial RNA. Unlabelled and ³²P-labelled bacteria were disrupted by grinding with alumina (Gros *et al.* 1961a) and total RNA was isolated from the crude extracts by the procedure of Monier, Naono, Hayes, Hayes & Gros (1962). Deoxyribonuclease, used in the RNA purification process, was freed of ribonuclease activity by chromatography on DEAE-cellulose (Polatnick & Bachrach, 1961). RNA preparations were stored as precipitates under ethanol at -15°. They are identified by their bacterial origin plus a numerical suffix, e.g. *E. coli* ML 308-1, *E. coli* B-1, *E. coli* B-2 etc. Fractionation of RNA was carried out by sedimentation on linear sucrose gradients (Gros *et al.* 1961a), volume 25 ml., prepared from 5% and 20% (w/v) solutions of sucrose in tris-HCl buffer, pH 7.4 (25 mM), or sodium acetate-acetic acid buffer, pH 4.5 (0.1 M), and which contained 0.1 M- or 0.6 M-NaCl and in some cases potassium polyvinyl sulphate (1 µg./ml.). The presence of potassium polyvinyl sulphate in sucrose gradients did not alter the sedimentation properties of RNA preparations but reduced the loss of stimulatory activity during sedimentation

(Table 1). Gradients were cooled to 3°, loaded with 1 ml. of freshly prepared solutions of RNA (1 mg./ml., pulse-labelled preparations; 4 mg./ml., unlabelled preparations) containing the same salt and buffer concentrations as those present in the gradients and centrifuged at 25000 rev./min. (average centrifugal force 64000g) for 14–16 hr. in a Spinco model L centrifuge (rotor SW 25) at refrigeration setting 0°. The maximum temperature observed in centrifuged gradients was 12°. After centrifugation gradients were fractionated at 4° (cold room) and their RNA distribution profiles obtained as described by Gros *et al.* (1961a).

Preparation of extracts. 'S 30' extracts were prepared from *E. coli* B as described by Soffer (1964). Several preparations of this extract were used. They differed little in protein content but markedly in activity and could be stored frozen (-5° to -10°) for several weeks without severe loss of activity.

Assays for amino acid incorporation. The standard incubation mixture (final vol. 0.5 ml.) contained the following components: tris-HCl buffer, pH 7.8, 19 µmoles; KCl, 12 µmoles; MgCl₂, 9 µmoles; ATP, 1.5 µmoles; GTP, 0.1 µmole; phosphoenolpyruvate (trisodium salt), 4 µmoles; 19 unlabelled L-amino acids, 0.05 µmole each; ¹⁴C-labelled L-amino acid, 0.025 µmole (sp. activity 6.5 µC/µmole); actinomycin D, 10 µg.; pyruvate kinase, 15 µg.; 'S 30' extract, 0.1 ml.

RNA preparations, ribonuclease and potassium polyvinyl sulphate were added as indicated.

After incubation for 45 min. at 37° reaction was stopped by the addition of 4 ml. of 5% (w/v) trichloroacetic acid. The resulting suspension was boiled for 20 min., centrifuged and the precipitate resuspended in 4 ml. of 5% trichloroacetic acid and filtered on membrane filters (HAWPO25; Millipore Filter Corp., Bedford, Mass., U.S.A.). Filters were washed with four 4 ml. portions of 5% trichloroacetic acid, dried and counted on a gas-flow counter.

RESULTS

Division of centrifuged sucrose gradients into fractions I, II and III. Fig. 1 shows sedimentation profiles obtained by centrifugation of a pulse-labelled bacterial RNA preparation on sucrose gradients containing 0.1 M- and 0.6 M-sodium chloride. Hayes *et al.* (1963) have referred to the three radioactive peaks in Fig. 1(b) as components I, II and III and we propose to use the same nomenclature here. Peaks I and II sediment a little faster than the 23s and 16s ribosomal RNA sub-units respectively. The division of such a gradient into regions corresponding to components I, II and III is made at points on the trailing edges of the 23s and 16s peaks. Typical sedimentation profiles obtained in the presence of 0.1 M- and 0.6 M-sodium chloride with the non-radioactive RNA preparations used in the present study are shown in Fig. 2. Such gradients are divided into three regions at points A and B whose positions were chosen as a result of the experiments with pulse-labelled RNA. Pooled RNA fractions obtained from several gradients (usually three to six)

Fig. 1. Sedimentation patterns of rapidly ^{32}P -labelled RNA (*E. coli* 200 PS-1, 1 mg., specific activity 2.5×10^5 counts/min./mg.) centrifuged for 14 hr. at $64000g_{av.}$ in the presence of 0.1M-NaCl (Fig. 1a) and 0.6M-NaCl (Fig. 1b). Gradients were buffered at pH 7.4 (25 mM-tris-HCl) and did not contain potassium polyvinyl sulphate. $\circ$, E_{260} m μ ; $\bullet$, trichloroacetic acid-precipitable radioactivity.

Fig. 2. Sedimentation profiles of bacterial RNA (*E. coli* B-1, 4 mg.) centrifuged for 15 hr. at $64000g_{av.}$ in the presence of 0.1M-NaCl (Fig. 2a) and 0.6M-NaCl (Fig. 2b). Gradients were buffered at pH 7.4 (25 mM-tris-HCl) and did not contain potassium polyvinyl sulphate.

were mixed with twice their volume of reagent-grade ethanol at 0° and the mixtures were allowed to precipitate for 30 min. at 0° . Nucleic acids were recovered by centrifugation and the pellets were recentrifuged to remove as much supernatant as possible and dissolved in tris-hydrochloride buffer, pH 7.4 (0.01M), at concentrations in the range 5–15 mg./ml. These solutions were frozen and stored at -5° .

General properties of the amino acid-incorporation system. The stimulation of amino acid incorporation by unfractionated RNA preparations was examined to determine some of the properties of the assay system. Figs. 3 and 4 show the time-course of the incorporation, and the sensitivity of the assay to addition of ribonuclease and actinomycin D. Although there was no evidence of an actinomycin D-inhibited DNA-dependent amino acid incorporation into protein, actinomycin D was included in all incubation mixtures.

The results shown in Fig. 5 demonstrate that the extent of amino acid incorporation is directly proportional to the amount of RNA added up to a limit which for the different preparations used varied from 0.3 mg. to 1.0 mg./assay volume (0.5 ml.). Further addition of RNA above this limit increased the amino acid incorporation slightly and did not have an inhibitory effect. The maximum stimulations observed in the linear range were approximately ten times the endogenous activity of the 'S 30' extracts.

Effect of sucrose-gradient sedimentation on the stimulatory activity of RNA preparations in vitro. The stimulatory activity of RNA preparations

Fig. 3. Time-course of amino acid incorporation *in vitro*. Standard amino acid-incorporation assay mixtures were: $\bullet$, unsupplemented; $\circ$, supplemented with 870 $\mu\text{g.}$ of RNA (*E. coli* B-3). Reactions were stopped at the time-intervals shown and incorporation of labelled amino acid was measured.

sedimented on sucrose gradients containing 0.1M- or 0.6M-sodium chloride can be recovered in 55-80% yield by ethanol precipitation. Recoveries calculated from the measured specific activities of the separated fractions I, II and III and the distribution of RNA in sedimented sucrose gradients fell in the same range. Comparison of the relative efficiencies of recovery after sedimentation in the presence of 0.1M- and 0.6M-sodium chloride revealed significant differences when the sedimentation media were buffered at pH 7.4 and did not

contain potassium polyvinyl sulphate, whereas in media buffered at pH 4.5 and containing potassium polyvinyl sulphate negligible differences were found (Table 1). The observation that sedimentation in media buffered at pH 4.5 and containing potassium polyvinyl sulphate gave higher recoveries of stimulatory activity suggested the presence of traces of ribonuclease in either the RNA preparations or the sedimentation media. This conclusion was supported by the finding that with sedimentation media buffered at pH 7.4 and not containing

Fig. 4. Effect of actinomycin D and of various concentrations of ribonuclease on the amino acid-incorporation assay *in vitro*. Assay mixtures were: ●, unsupplemented, in the absence of actinomycin D; ■, unsupplemented in the presence of actinomycin D (10 μg.); ○, supplemented with *E. coli* ML 308-1 RNA (455 μg.) in the absence of actinomycin D; □, supplemented with *E. coli* ML 308-1 RNA (455 μg.) in the presence of actinomycin D (10 μg.).

Fig. 5. Variation of incorporation with the quantity of RNA assayed. Assays were carried out in the range 0-1500 μg. of added RNA with the following preparations: ○, *E. coli* ML 308-1; ●, △, □, *E. coli* B-1, -2 and -3 respectively.

Table 1. *Effect of sedimentation in different media on the specific stimulatory activity of RNA preparations*

By using the standard amino acid-incorporation assay, the specific stimulatory activities (μmoles of L-threonine incorporated/mg. of RNA) of several RNA samples were measured before and after sucrose gradient sedimentation. Centrifuged gradients containing *E. coli* B-3 RNA were poured into 2 vol. (52 ml.) of ethanol at 0° and the precipitated unfractionated RNA was collected by centrifugation and assayed directly. Results for the other RNA preparations were calculated from the measured specific activities of the separated fractions I, II and III, and the observed u.v. distribution in the gradients. KPVS, Potassium polyvinyl sulphate.

RNA preparation	Specific stimulatory activity				
	Before sedimentation	After sedimentation			
		In NaCl (0.1M), pH 7.4	In NaCl (0.6M), pH 7.4	In NaCl (0.1M), KPVS (1 μg./ml.), pH 4.5	In NaCl (0.6M), KPVS (1 μg./ml.), pH 4.5
<i>E. coli</i> ML 308	2900	1100	2300	—	—
<i>E. coli</i> B-1	2100	350	920	—	—
<i>E. coli</i> B-2	1600	850	1000	—	—
<i>E. coli</i> B-3	600	—	—	650	380
<i>E. coli</i> B-4	300	—	—	230	230

Table 2. *Inhibitory effect of potassium polyvinyl sulphate in the amino acid-incorporation assay system*

Expt. 1. Increasing amounts of potassium polyvinyl sulphate (KPVS) were added to standard assay mixtures containing a constant quantity of RNA (preparation *E. coli* B-1). Expt. 2. RNA samples (preparation *E. coli* B-1) were dissolved in the solutions listed, at 0.4 mg./ml., recovered by ethanol precipitation (2 vol. of ethanol, 30 min. at 0°), redissolved in tris-HCl buffer, pH 7.8 (5 mM), at 2.3 mg./ml., and their stimulatory activities assayed.

Expt. 1	RNA (μg.)	KPVS (μg.)	L-[¹⁴ C]Proline
			incorporated (μmoles)
	0	0	42
	230	0	168
	230	0.1	173
	230	1.0	180
	230	5.0	165
	230	12.5	43
	230	25.0	23

Expt. 2	RNA (μg.)	Solvent for ethanol precipitation	L-[¹⁴ C]Proline
			incorporated (μmoles)
	0	—	65
	230	Unprecipitated	230
	230	(A) Sodium acetate-acetic acid, pH 4.5 (0.01 M); NaCl (0.1 M)	160
	230	Solvent A plus KPVS (100 μg./ml.)	27
	230	(B) Tris-HCl, pH 7.4 (25 mM); NaCl (0.1 M)	345
	230	Solvent B plus KPVS (100 μg./ml.)	27

potassium polyvinyl sulphate higher recoveries were observed in 0.6 M- than in 0.1 M-sodium chloride (Table 1). High concentrations of salt are known to inhibit ribonuclease action (Hayes *et al.* 1963).

The routine use of potassium polyvinyl sulphate in sucrose gradients necessitated an examination of its effect in the amino acid-incorporation assay system *in vitro*. It was found that this material is an inhibitor of amino acid incorporation at high concentrations and that it is precipitated to some extent with RNA when the latter is concentrated by ethanol precipitation (Table 2). For this reason potassium polyvinyl sulphate was added to sedimentation media at a low concentration (1 μg./ml.). The relevance of the presence of traces of nuclease activity in the RNA preparations or sedimentation media to the interpretation of the experimental results is discussed below.

Stimulatory activity of RNA fractions in the amino acid-incorporation assay in vitro. After the demonstration that the property of stimulating amino acid incorporation could be recovered in good yield and assayed satisfactorily when RNA preparations were subjected to sucrose-gradient centrifugation followed by ethanol precipitation, the stimulatory properties of the RNA fractions thus obtained were studied. Stimulation of amino acid incorporation by fraction I (0.6 M-sodium chloride) and fraction II (0.1 M- and 0.6 M-sodium chloride) was propor-

Fig. 6. Stimulatory activities of fractions I, II and III isolated from *E. coli* B-2 RNA in the presence of 0.1 M- and 0.6 M-NaCl. The standard assay system was used. ●, Fraction I, 0.1 M-NaCl; ○, fraction I, 0.6 M-NaCl. ▲, Fraction II, 0.1 M-NaCl; △, fraction II, 0.6 M-NaCl. ■, Fraction III, 0.1 M-NaCl; □, fraction III, 0.6 M-NaCl.

tional to the amount of added RNA within the ranges tested. For fraction III (0.1M- and 0.6M-sodium chloride) proportionality extended to 0.125 and 0.1mg. of added RNA respectively (Fig. 6). Fraction I isolated after sedimentation of RNA on gradients containing 0.1M-sodium chloride always had a very low activity which was not proportional to the amount of material assayed at the amounts of added RNA used. Small but probably significant stimulations were always observed with the smallest amounts (100 μ g.) of this fraction assayed whereas with larger amounts (200–400 μ g.) stimulations were much diminished and were usually too low to be significant. Calculations of the specific activity of this fraction were made from stimulations observed with the smallest amount assayed. These stimulations were usually smaller than the activity of the unsupplemented 'S 30' extract and so the derived specific activities must be regarded as no more than approximations. The results of amino acid-incorporation assays may be expressed graphically (Fig. 6) or numerically as the specific activities of the isolated fractions I, II and III. Alternatively, by using these specific activity values and the observed distribution of RNA in sucrose gradients the percentage of the total stimulatory activity found in fractions I, II and III may be calculated. Specific activities and activity distributions derived from four experiments

are combined in Table 3. These results show that an important redistribution of stimulatory activity is produced when RNA preparations are centrifuged in the presence of 0.6M-sodium chloride. This finding may be compared with the similar redistribution of radioactivity observed when pulse-labelled RNA preparations are centrifuged on sucrose gradients containing 0.6M-sodium chloride (Hayes *et al.* 1963, 1966). Calculations of the percentage of the total acid-precipitable radioactivity found in the three regions of a sucrose gradient defined above are given for several pulse-labelled preparations in Table 4. Several explanations of the effect of 0.6M-sodium chloride on the sedimentation properties of stimulatory RNA are possible. A real redistribution of stimulatory activity is consistent with the observed phenomenon but an apparent redistribution of biological activity could be produced either by demasking of latent activity in the 20–30s region of sucrose gradients or by the displacement of an inhibitory principle from regions of high to regions of low sedimentation constant, both effects being ionic strength-dependent. Experiments were therefore carried out to test these possibilities.

When measurements were made of the specific activities of fractions I, II and III isolated in 0.1M-sodium chloride and of mixtures of equal amounts of fractions I and II, and of fractions II

Table 3. *Total and specific stimulatory activities of RNA fractions obtained by sedimentation in the presence of high and low concentrations of sodium chloride*

RNA preparations listed were fractionated as described in the Materials and Methods section and the isolated fractions assayed. Results are expressed as μ moles of L-[14 C]threonine incorporated/mg. of RNA (specific activities) and as percentages of the sum of the total stimulatory activities recovered in fractions I, II and III in each case. KPVS, Potassium polyvinyl sulphate.

RNA preparation		Fractionation media and stimulatory activities							
		NaCl (0.1 M), pH 7.4		NaCl (0.6 M), pH 7.4		NaCl (0.1 M), KPVS (1 μ g./ml.), pH 4.5		NaCl (0.6 M), KPVS (1 μ g./ml.), pH 4.5	
		Specific activity	% of total activity	Specific activity	% of total activity	Specific activity	% of total activity	Specific activity	% of total activity
Fraction									
<i>E. coli</i> ML 308	I	270	3	1900	29				
	II	400	12	2200	24	—	—	—	—
	III	2480	85	3000	47				
<i>E. coli</i> B-2	I	80	4	640	32				
	II	390	19	510	16	—	—	—	—
	III	2700	77	2000	52				
<i>E. coli</i> B-3	I	0	0	920	37				
	II	90	10	420	16	—	—	—	—
	III	850	90	1230	47				
<i>E. coli</i> B-4	I					10	2	380	30
	II	—	—	—	—	170	20	150	28
	III					570	78	490	42

Table 4. *Distribution of radioactivity in sedimented sucrose gradients loaded with pulse-labelled RNA preparations*

Pulse-labelled RNA preparations were fractionated by sedimentation on sucrose gradients containing high (0.6M) and low (0.1M) concentrations of NaCl. Results are expressed as the percentage of the total trichloroacetic acid-precipitable radioactivity present in the centrifuged gradients which was found in fractions I, II and III as defined in Fig. 1.

RNA preparation		Percentage radioactivity distribution	
	Fraction	In NaCl (0.1M), pH 7.4	In NaCl (0.6M), pH 7.4
<i>E. coli</i> 200 PS-1	I	4	33
	II	14	25
	III	82	42
<i>E. coli</i> 200 PS-2	I	8	18
	II	23	30
	III	69	52
<i>E. coli</i> 200 PS-3	I	11	27
	II	18	24
	III	71	49
<i>E. coli</i> 200 PS-4	I	23	46
	II	22	18
	III	55	36

Table 5. *Possible existence of inhibitory activity in RNA fractions I and II isolated at low ionic strength*

RNA preparation *E. coli* B-2 was fractionated on sucrose gradients at pH 7.4 in the presence of 0.1M-NaCl. The isolated fractions were assayed for stimulatory activity alone and after mixing equal weights of fractions I and III and II and III. Figures in parentheses are the sums of stimulatory activities observed with single fractions at the levels at which each was present in the assay of their mixture. The RNA preparation *E. coli* B-2 displayed a linear relationship between quantity and stimulatory activity up to at least 500 μ g. of RNA (see Fig. 5). In the Table the endogenous activity (127 μ moles of L-[¹⁴C]-threonine) of the 'S 30' extract has been subtracted throughout.

RNA fraction	Quantity (μ g.)	L-[¹⁴ C]Threonine incorporated (μ moles)
I	114	9
	228	0.8
II	114	49
	228	70
III	114	308
	228	480
I+III (1:1)	228	208 (317)
	456	252 (481)
II+III (1:1)	228	247 (357)
	456	408 (550)

being the more active in this respect. The nature and importance of this inhibitory activity are discussed below.

To examine the possibility of the existence of a demasking effect the following experiment was carried out. An RNA preparation was fractionated on gradients containing 0.1M- and 0.6M-sodium chloride and the activities of the various fractions were assayed. Mixtures were then prepared of equal amounts of fractions I and II, and of fractions I, II and III isolated on gradients containing 0.1M-sodium chloride. These mixtures were refractionated on gradients containing 0.6M-sodium chloride and the fractions thus obtained were assayed. Table 6 summarizes the results of this experiment. Although the successive sedimentation and precipitation steps are accompanied by decreases in the specific activities of the three fractions (compare I_B, II_B, III_B and I_C, II_C, III_C) the activity distributions do not change significantly. A comparison of the specific activities of fractions I_A, II_A, III_A, I_C, II_C, III_C, and I_D, II_D, shows that I_D and II_D are much less active than fractions I_C and II_C and that the activity per milligram of the combined fractions I_D+II_D is lower than that of the combined fractions I_A+II_A, whereas fractions I_C+II_C together contain more activity per milligram than I_A+II_A. Hence the increased activities of fractions I and II isolated in 0.6M-sodium chloride compared with the activities of the corresponding fractions isolated in 0.1M-sodium chloride are due to a transfer of activity from fraction III to fractions I and II. This transfer

and III, the results given in Table 5 were obtained. They suggest that fractions I and II possess some inhibitory activity in the assay system, fraction I

Table 6. *Demonstration of the transfer of stimulatory activity from fractions II and III to fraction I of sucrose gradients during sedimentation of RNA preparations in high ionic strength medium*

RNA preparation ML 308-1 was fractionated in the presence of NaCl at 0.1M (fractions I_A, II_A, III_A) and 0.6M (fractions I_B, II_B, III_B). Mixtures were prepared containing equal weights of fractions I_A, II_A and III_A and of fractions I_B and II_B. These mixtures were then refractionated in the presence of NaCl at 0.6M, yielding respectively fractions I_C, II_C, III_C and fractions I_D and II_D. The 11 fractions were assayed for stimulatory activity and from the results obtained and the measured RNA distributions in the various sucrose gradients the percentage distributions of total stimulatory activity were calculated.

RNA preparation	Concn. of NaCl in sucrose gradient (M)	RNA fraction assayed (μg.)		Specific stimulatory activity (μmoles of L-[¹⁴ C]threonine incorporated/mg. of RNA fraction)	Percentage distribution of stimulatory activity in gradients
ML 308-1	0.1	I _A	250	52	8
			500	51	
		II _A	192	280	27
			384	270	
		III _A	226	600	65
ML 308-1	0.6	I _B	452	540	
			246	390	42
		II _B	492	320	
			163	250	18
		III _B	326	220	
ML 308-1 fractions IA + IIA + IIIA (1:1:1)	0.6	I _C	173	500	40
			346	400	
		II _C	109	190	43
			218	210	
		III _C	120	220	24
ML 308-1 fractions IA + IIA (1:1)	0.6	I _D	240	210	
			105	270	
		II _D	210	250	90
			180	120	
			360	120	10
			197	22	
			394	0.4	

is accompanied by a decrease in the specific activity of fraction III (compare III_A and III_B, Table 6).

DISCUSSION

It appears from data presented here (Table 4) and by others (Willson & Gros, 1964) that ribosomal RNA 23s (and perhaps 16s) sub-units possess some inhibitory activity in the assay system *in vitro*. These findings contrast with the observation that rat-liver nuclear ribosomal RNA is devoid of inhibitory activity in a system *in vitro* stimulated by rat-liver nuclear template RNA (Brawerman, Gold & Eisenstadt, 1963).

The explanation of this inhibitory effect as due to a non-specific binding of ribosomal RNA to ribosomes which would then be unable to interact with messenger RNA must be rejected since such binding is not observed to take place (Gros *et al.* 1961a). An analogous explanation based on complex-formation between messenger and ribo-

somal RNA must also be discarded since though such complexes can be formed they are stable only at temperatures below approx. 16° (Hayes *et al.* 1966). Previous studies (Willson & Gros, 1964) showed that in fact the radioactive material present in the region of a sucrose gradient defined as fraction I (0.1M-sodium chloride) forms complexes with ribosomes in excellent yield. There remain two possible explanations for this slight inhibitory effect. First, ribosomal RNA possesses polyanionic properties, e.g. capacity to bind cations, and basic proteins, similar to those possessed by polyvinyl sulphate, which is an inhibitor of the assay system at high concentrations. It seems possible that the inhibitory action of ribosomal RNA might be the result of such properties. Secondly, the possibility that traces of ribonuclease are bound to ribosomal RNA in the RNA preparations cannot be excluded. Observations described above of the effect of ribonuclease inhibitors in improving the recovery of stimulatory activity from sucrose gradients,

indicate that ribonuclease contamination, of either the RNA preparations or the sedimentation media, exists. However, the distributions of rapidly labelled RNA and of stimulatory activity in centrifuged sucrose gradients resemble each other closely in both high and low ionic strength media. This suggests two possibilities. First, the degradative activity may be uniformly distributed in sucrose gradients in both high and low ionic strength media, which would be the case if it were for example a contaminant of the sucrose used. Secondly, the degradative activity may be present in such small amounts that even when it is non-uniformly distributed a significant differential effect is not observed. It may be concluded that the results presented here are not artifacts due to the presence of a ribonuclease contaminating the RNA preparations or the sedimentation media. The effect of a sedimentation medium containing 0.6M-sodium chloride on the distribution of stimulatory activity in sucrose gradients is therefore due to a real transfer of active material from regions of low to regions of high sedimentation coefficient. This transfer of activity is closely paralleled by the transfer of pulse-labelled RNA observed under the same conditions. Data of a similar nature but in part obtained by a different fractionation process have been presented previously (Gros, Naono, Woese, Willson & Attardi, 1962; Willson & Gros, 1964). In these studies RNA was fractionated in the presence of 0.1M-sodium chloride on sucrose gradients and at high ionic strength (0.45–0.65M-sodium chloride approximately; Monier *et al.* 1962) on columns of kieselguhr coated with methylated serum albumin (Mandell & Hershey, 1960). It was observed in both types of fractionation that pulse-labelled material and stimulatory activity were always found together. It is now known (Hayes *et al.* 1966) that the fractionation of pulse-labelled RNA obtained by column chromatography on kieselguhr coated with methylated albumin is the same as that produced by sedimentation on sucrose gradients containing 0.6M-sodium chloride.

The present study and the previous work of a similar nature discussed above support the general conclusion that the component of RNA preparations responsible for the stimulation *in vitro* of amino acid incorporation into acid-insoluble peptides is contained by their rapidly labelled fraction.

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