Inhibition by Soluble Ribonucleic Acid of Stimulatory Effect of Liver Template Ribonucleic Acid

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1. Liver soluble RNA (s-RNA) and, to a smaller extent, *Escherichia coli* s-RNA inhibited the stimulation of [^{14}C]leucine incorporation into protein in an *E. coli* S-30 system brought about by liver microsomal RNA or polyuridylic acid as template. 2. The inhibitory activity was associated with the fraction of the s-RNA possessing transfer-RNA activity. 3. The inhibition was exercised at a stage after charging of the s-RNA with amino acid. 4. Neither the method of preparation of the s-RNA nor its state of amino acid acylation affected its inhibitory action. 5. Stimulation of [^{14}C]phenylalanine incorporation by polyuridylic acid or by liver microsomal RNA was not inhibited by addition of s-RNA. 6. It appears that excess of s-RNA inhibits the ambiguous incorporation of leucine with polyuridylic acid and also that something similar occurs with a natural template. 7. Estimation of messenger activity of samples of RNA should be carried out only after removal of s-RNA.

The *Escherichia coli* cell-free RNA-dependent amino acid-incorporating system devised by Nirenberg & Matthaei (1961) has been used extensively to assay the messenger activity of samples of RNA extracted from a wide range of tissues.

During preliminary experiments, before using the system to assay the template activities of RNA samples from mammalian tissues, we noticed that the s-RNA† fraction of rat liver inhibited the stimulation of amino acid incorporation into protein brought about by addition of liver microsomal RNA to the *E. coli* ribosomal system. This phenomenon was investigated. During the work it became apparent that inhibition by added s-RNA was obtained when leucine but not when phenylalanine was used as precursor for polypeptide synthesis with both liver microsomal RNA and polyU as template material. This suggested that our findings might be related to those of Grunberg-Manago & Dondon (1965), So & Davie (1965) and Pestka, Marshall & Nirenberg (1965), who reported that various agents, including s-RNA, depressed the incorporation into polypeptide of amino acids other than phenylalanine in the presence of polyU.

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† Abbreviations: s-RNA, soluble RNA; polyU, polyuridylic acid.

MATERIALS AND METHODS

Materials. L-[U- ^{14}C]Phenylalanine (34 mc/m-mole), L-[U- ^{14}C]leucine (163 mc/m-mole) and ^{14}C -labelled algal-protein hydrolysate (200 μC /mg.) were purchased from The Radiochemical Centre (Amersham, Bucks.). PolyU was from Miles Chemical Co. (Elkart, Ind., U.S.A.). Tris and the disodium salts of ATP and GTP were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Creatine phosphate and creatine phosphokinase were obtained from British Drug Houses Ltd. (Poole, Dorset) and C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany) respectively.

Preparation of liver cell fractions. Albino rats, bred in the laboratory, were starved overnight to deplete the liver of glycogen, decapitated and bled. The liver was rapidly removed and cut up with scissors in ice-cold 0.25 M-sucrose–10 mM-magnesium acetate–20 mM-tris buffer, pH 7.8. The liver was homogenized, centrifuged at 10000g for 10 min. at 0° and the supernatant material centrifuged at 100000g for 2 hr. at 0° to separate microsomes from cell sap as described by Munro, Jackson & Korner (1964).

Extraction of RNA. RNA was extracted from the unfractionated homogenate, from the 10000g supernatant material, from the microsome pellet and from the cell sap left unsedimented after 2 hr. centrifugation at 100000g by the phenol-sodium dodecyl sulphate method (Munro *et al.* 1964). At least three extractions with phenol were employed.

The RNA was precipitated with 2% (w/v) sodium acetate, pH 5.9, and 3 vol. of cold ethanol, washed with ethanol, redissolved in 0.02 M-sodium acetate buffer, pH 7.8, and

dialysed at 4° for 6 hr. against repeatedly changed acetate buffer. The dialysis tubing had been boiled in EDTA before use.

Some RNA samples were treated with a final concentration of 2M-LiCl to remove low-molecular-weight RNA (Barlow, Mathias, Williamson & Gammack, 1963). Ethanol precipitation of the material that remained in solution after LiCl treatment yielded low-molecular-weight RNA.

Preparation of the *E. coli* S-30 system. The *E. coli* S-30 system was prepared essentially as described by Nirenberg (1964) except that the medium employed was 20mM-magnesium acetate-100mM-NH₄Cl-6mM-mercaptoethanol-20mM-tris buffer, pH 7.8. The *E. coli* S-30 system was incubated at 37° for 80 min. in the presence of 5mM-ATP before storage at -20°.

The assay of messenger activity was carried out as described by Nirenberg (1964). The reaction was stopped by addition of 2 ml. of 0.5N-HClO₄ containing unlabelled amino acid corresponding to the labelled one used. The precipitated protein was separated by centrifuging, dissolved in N-NaOH containing unlabelled amino acid and incubated for 1 hr. at 37° to hydrolyse RNA (Munro *et al.* 1964). Then 2N-HClO₄ containing unlabelled amino acid was added and the reprecipitated protein was filtered on to membrane filters and washed with 0.05N-HClO₄ containing unlabelled amino acid. The filters were dried in an oven at 60° and fixed to cardboard disks with rubber cement and their radioactivity was assessed with a Nuclear-Chicago gas-flow counter (efficiency about 10%). RNA was estimated as described by Munro *et al.* (1964).

Charging of s-RNA with amino acids in vitro. The s-RNA of *E. coli* was charged with radioactive amino acids by incubation of *E. coli* 100000g supernatant material, supplemented with *E. coli* s-RNA, in the reaction mixture described by Nirenberg (1964) except that GTP was omitted. Since no ribosomes are present the product should be s-RNA charged with leucine. The reaction was stopped, after 10 min. incubation, by adding ice-cold 5% (w/v) trichloroacetic acid. The precipitated protein and RNA was filtered on to membranes and washed with cold 5% trichloroacetic acid and the radioactivity measured as described above. As a control, some samples were incubated at 37° with N-NaOH for 1 hr. to show that all the radioactive amino acid measured had been associated with RNA and not with protein.

Fractionation of s-RNA. The RNA extracted from 100000g supernatant material was passed through a column (30 cm. long × 1.1 cm.² cross-sectional area) of Sephadex G-100 (bed vol. approx. 100 ml.) and eluted with 2mM-EDTA-0.2M-NaCl-0.02M-tris buffer, pH 7.4. Two peaks (Fig. 3) of material with a maximum absorption at 260 mμ were collected, reprecipitated with ethanol and dialysed against 0.02M-tris buffer, pH 7.8. The peak for the fraction with lower molecular weight (S₂) was eluted from the column at a position that would be expected of material with molecular weight 20000-30000.

RESULTS

The *E. coli* S-30 system was shown to be active by incubating it with polyU, in that incorporation of [¹⁴C]phenylalanine into proteins was greatly stimulated by polyU and about 10 μg. of polyU

was found to saturate the amount of *E. coli* S-30 preparation used. The reaction was complete within 15 min. The incorporation of the *E. coli* S-30 fraction in the absence of added RNA was too high to be used with mammalian RNA as template unless the *E. coli* S-30 fraction had been pre-incubated as described in the Materials and Methods section, and so this was done as a routine.

A typical result obtained on adding various amounts of total liver RNA and liver microsomal RNA to the *E. coli* S-30 system is shown in Fig. 1. Stimulation of leucine incorporation was obtained on addition of both samples of liver RNA, but when increasing amounts of RNA were added an inhibitory effect predominated. The stimulation by total RNA was much less than that by microsomal RNA.

It seemed possible that low-molecular-weight RNA might be the inhibitory material, for s-RNA and oligonucleotides had been reported to inhibit amino acid activation (Barth & Hele, 1964). Total and microsomal RNA were therefore prepared by the lithium chloride method to remove low-molecular-weight RNA (Barlow *et al.* 1963). The absence of s-RNA was verified by sucrose-density-gradient analysis, which showed almost no material sedimenting at 4s. When the high-molecular-weight RNA samples were assayed in the *E. coli* S-30 system, the inhibitor appeared to have been removed with the elimination of s-RNA (Fig. 1).

The s-RNA was extracted from the lithium chloride-soluble material as described in the Materials and Methods section and added to the

Fig. 1. Stimulation of [¹⁴C]leucine incorporation in the *E. coli* S-30 system by the presence of total RNA of rat liver (□), microsomal RNA (Δ) and microsomal RNA treated with LiCl to remove low-molecular-weight RNA (○).

E. coli S-30 system to supplement the lithium chloride-treated microsomal RNA. The amounts of s-RNA mixed with microsomal RNA were one-tenth, one-quarter or one-third of the total RNA added to the system. The results are shown in Fig. 2 and it is clear that the s-RNA fraction contains the inhibitor. It should be mentioned that liver s-RNA added to the *E. coli* S-30 system in the absence of microsomal RNA did not inhibit leucine incorporation: indeed it sometimes caused some stimulation.

Table 1 records the effect, on the stimulation of leucine incorporation by microsomal RNA, of adding s-RNA prepared from the 100 000g supernatant material by the phenol method. This material is as effective an inhibitor of stimulation as the s-RNA prepared from the lithium chloride-soluble fraction of microsomal RNA. Table 1 also shows that the s-RNA prepared from 100 000g supernatant material after it had been passed through a Sephadex G-25 column to remove inhibitors of amino acid incorporation (Munro *et al.* 1964) also had unimpaired inhibitory activity in the *E. coli* S-30 system. Aminoacylated s-RNA and deacylated s-RNA inhibit the microsomal stimulation of leucine incorporation to about the same extent (Table 1). Also, *E. coli* s-RNA did show some inhibition of the stimulation of leucine incorporation by microsomal RNA (Table 1). The stimulation shown by the *E. coli* S-30 system on adding *E. coli* s-RNA in the absence of microsomal RNA was greater than with liver s-RNA and the inhibition noted was much smaller.

Table 2 shows the results of an experiment in which the charging of the *E. coli* s-RNA by radioactive amino acids was assessed in the presence of various amounts of liver s-RNA. It is clear that the inhibitory action of the liver s-RNA cannot be explained in terms of interference with the activa-

tion of amino acids or their transfer to s-RNA to form aminoacyl-s-RNA complexes, for the added liver RNA did not affect the charging of the s-RNA of *E. coli*.

This conclusion is supported by the observation that incorporation into polypeptide of leucine from leucyl-s-RNA is inhibited by the addition of liver s-RNA.

Not all the s-RNA fraction is transfer RNA. Transfer activity could be separated from the rest of the s-RNA fraction (probably fragments of ribosomal RNA) as described in the Materials and Methods section (Fig. 3). The RNA of the second peak (S_2) was found to have about five times as much specific transfer-RNA activity as the RNA of the first peak (S_1). When the ability of the two components to inhibit leucine incorporation in the presence of ribosomal RNA was tested (Fig. 4) it was found that the inhibitory activity resided in the transfer-RNA fraction. The other RNA peak had no inhibitory activity.

It was noted (Table 3) that when microsomal RNA and polyU were added together to the *E. coli* S-30 system the incorporation of phenylalanine, leucine and algal-protein hydrolysate was much greater than the sum of the incorporations obtained when polyU and microsomal RNA were added separately to the *E. coli* S-30 system. With

Fig. 2. Stimulation of [^{14}C]leucine incorporation in the *E. coli* S-30 system by the presence of rat-liver LiCl-treated microsomal RNA (○) supplemented with 10% (Δ), 25% (□) or 33.3% (●) of s-RNA.

Table 1. Effect of source of s-RNA, and of various treatments of it, on its ability to inhibit the stimulation of [^{14}C]leucine incorporation by liver microsomal RNA

The *E. coli* S-30 system (about 1.5 mg. of protein and 0.15 mg. of RNA) was incubated for 1 hr. in 20 mM-tris buffer, pH 7.8, containing magnesium acetate (20 mM), NH_4Cl (100 mM), mercaptoethanol (6 mM), ATP (0.25 mM), GTP (0.05 mM), creatine phosphate (0.5 mg.), creatine phosphokinase (10 $\mu\text{g.}$) and [^{14}C]leucine (0.06 μC) with 300 $\mu\text{g.}$ of liver microsomal RNA. The final volume was 0.25 ml., and 80 $\mu\text{g.}$ of the various s-RNA preparations was added.

Type of s-RNA added	Incorporation of [^{14}C]leucine into protein, corrected for incorporation obtained in the absence of added template RNA (counts/min.)
None	850
LiCl supernatant s-RNA	360
100 000g supernatant s-RNA	310
100 000g Sephadex-treated supernatant s-RNA	380
LiCl supernatant s-RNA stripped of amino acids	410
<i>E. coli</i> s-RNA	635

Table 2. Influence of rat-liver s-RNA on charging by [¹⁴C]leucine of *E. coli* s-RNA in the *E. coli* S-30 system supplemented with *E. coli* s-RNA

E. coli 100000g supernatant material, supplemented with the stated amounts of *E. coli* and liver s-RNA, was incubated for 10min. in the medium described in the legend to Table 1 except that GTP was omitted. The reaction was stopped after 10min. with ice-cold 5% (w/v) trichloroacetic acid.

<i>E. coli</i> s-RNA added (μg.)	Incorporation of [¹⁴ C]leucine (counts/min.) into cold-trichloroacetic acid-precipitable material				
	Liver s-RNA added (μg.) ...	0	20	50	100
0		672	769	734	789
20		981	1037	1003	1021
40		1394	1368	1325	1332
80		1884	1849	1782	1786
200		3271	3529	3579	3508

Fig. 3. Extinction at 260mμ of eluate from a Sephadex G-100 column of liver s-RNA. Elution was with 2mm-EDTA-0.2M-NaCl-0.02M-tris buffer, pH7.4.

Fig. 4. Stimulation of [¹⁴C]leucine incorporation in the *E. coli* S-30 system by LiCl-treated liver microsomal RNA (○), in the presence of the S₁ peak (see Fig. 3) (△) and in the presence of the S₂ peak (see Fig. 3) (□). The S₂ peak had five times the transfer-RNA activity of the S₁ peak.

phenylalanine, the observed incorporation was 2.4 times that expected whereas with leucine it was four times as great.

Table 4 shows the results of experiments in which polyU was used as a stimulator of amino acid incorporation in place of microsomal RNA. The s-RNA of liver and *E. coli* did not inhibit the polyU-stimulated incorporation of phenylalanine: indeed they stimulated it, confirming the finding of So & Davie (1965) (cf. Nirenberg & Matthaei, 1961). When the stimulation of leucine incorporation by added polyU was studied, however, the inhibition by s-RNA was again evident. The inhibition by liver s-RNA was more marked than that by *E. coli* s-RNA. Inhibition of incorporation was also noted when an algal-protein hydrolysate was used as the source of radioactive amino acids.

Table 4 also shows that incorporation of amino

acids from acylated s-RNA is inhibited by liver s-RNA when leucyl-s-RNA is used but very little inhibition is seen when phenylalanyl-s-RNA is used in the presence of polyU.

It appeared, then, that the inhibition of leucine incorporation by s-RNA in the presence of polyU was related to the reports of other workers (Grunberg-Manago & Dondon, 1965; So & Davie, 1965; Pestka *et al.* 1965) on s-RNA depression of ambiguity towards polyU. However, these workers had demonstrated a depression of the ambiguity toward polyU for isoleucine and not leucine (So & Davie, 1965). Further, as shown in Table 4, the results showed a depression of leucine incorporation

Table 3. *Enhanced stimulatory activity for amino acid incorporation in the E. coli S-30 system when polyU and microsomal RNA are present together as templates*

The specific activities of the precursors were widely different (see the Materials and Methods section). The conditions of incubation are as described in the legend to Table 1.

Radioactive amino acid(s) used	Stimulation of amino acid incorporation into protein (counts/min.)		
	With microsomal RNA (300 μ g.)	With polyU (20 μ g.)	With microsomal RNA (300 μ g.) and polyU (20 μ g.)
Phenylalanine	80	3300	8000
Leucine	450	4550	19900
Algal-protein hydrolysate	375	1450	3800

Table 4. *Effect of liver s-RNA on stimulation of amino acid incorporation in the E. coli S-30 system with polyU and microsomal RNA as template*

The specific activities of the precursor were widely different (see the Materials and Methods section). Incubation conditions were as described in the legend to Table 1; 20 μ g. of polyU or 350 μ g. of microsomal RNA was added as indicated, and 70 μ g. of s-RNA was added where shown.

		Stimulation of amino acid incorporation (counts/min.)				
s-RNA	Template RNA	[¹⁴ C]Phenyl-alanine	[¹⁴ C]Leucine	¹⁴ C-labelled algal-protein hydrolysate	[¹⁴ C]Phenyl-alanyl-s-RNA	[¹⁴ C]Leucyl-s-RNA
—	—	26	44	253	21	31
—	PolyU	3340	4585	1473	285	425
Liver	—	60	130	271	—	—
Liver	PolyU	4981	3090	1317	272	295
<i>E. coli</i>	—	49	219	224	—	—
<i>E. coli</i>	PolyU	4174	3776	1256	—	—
—	Liver microsomal	377	885	—	—	—
Liver	Liver microsomal	350	565	—	—	—

by added s-RNA when rat-liver RNA was the template as well as when polyU was the template. We have also noted inhibition by added s-RNA of stimulation of incorporation of several amino acids (glycine, valine, lysine, proline and tyrosine) in the presence of liver microsomal RNA.

DISCUSSION

The presented results show that the s-RNA fraction of rat liver inhibits the stimulation of amino acid incorporation seen when liver high-molecular-weight RNA is used as template in the *E. coli* S-30 system.

The inhibitory material is either transfer RNA or material that accompanies it during chromatography on Sephadex G-100 (Fig. 3). RNA in the cell sap, which does not have transfer activity, did not inhibit leucine incorporation (Fig. 4). It should be mentioned that Richards, Coll & Gratzner (1965) have noted that s-RNA contains components, not separable by Sephadex G-100, lacking transfer

activity. The results indicate that neither the method of preparation of the RNA nor the prior purification of the cell sap, from which it is isolated, by passage through a column of Sephadex G-25 (Munro *et al.* 1964) altered the inhibitory activity (Table 1). Transfer RNA was found to be inhibitory whether charged with amino acid or not (Table 1). The inhibition of amino acid activation by s-RNA and oligonucleotides reported by Barth & Hele (1964) cannot be the explanation of the effects we have observed, for inhibition occurs, not at the activation step, but at a point after charging of s-RNA (Table 2).

Di Girolamo, Henshaw & Hiatt (1964) reported that deoxyribonucleotides, present as contaminants of preparations of nuclear RNA, inhibited the stimulatory activity of the RNA. Deoxyoligonucleotides may have been present in our preparations of the total liver RNA but were absent from the microsomal and s-RNA fractions.

Di Girolamo *et al.* (1964) have reported results of experiments in which RNA fractions after sucrose-

density-gradient centrifugation were assayed for messenger activity in the S-30 system. Template activity was found to be associated mainly with the 19s RNA. However, the inhibitory nature of s-RNA could obscure messenger activity of samples taken from the top of the gradient unless the s-RNA had been removed from the material before assay. If this is not done a false distribution of messenger activity and of its sedimentation coefficients might be obtained.

Recently a number of reports have appeared on the alteration of coding ambiguity to polyU by organic solvents, salts, temperature, streptomycin and amino acids. In addition, Grunberg-Manago & Dondon (1965) showed that *E. coli* s-RNA decreased the ambiguous incorporation of serine and isoleucine when polyU was used as template but not when an adenylic acid-uridylic acid or uridylic acid-cytidylic acid co-polymer was used as template. Pestka *et al.* (1965) found that at low s-RNA concentrations polyU directed the binding to ribosomes of most of the available [¹⁴C]isoleucyl-s-RNA but that less was bound at higher s-RNA concentrations. So & Davie (1965) also reported decreased isoleucine incorporation with polyU as template when s-RNA, in excess of that usually added to provide transfer activity, was present. They also mentioned that the ambiguity of leucine incorporation was not affected by the presence of the extra s-RNA. We found that *E. coli* s-RNA did, to some extent, inhibit polyU-directed leucine incorporation. The inhibition was, however, less marked than when liver s-RNA was used.

The hypothesis has been advanced (Grunberg-Manago & Michelson, 1964; So & Davie, 1965) that a tautomeric shift of uracil might explain at least the ethanol-induced ambiguity. In this view the enol form of uracil could act as complementary base to the keto form of uracil or to guanine as well as to adenine. This hypothesis does not explain how alterations in ambiguity could be brought about by addition of excess of s-RNA.

Pestka *et al.* (1965) have explained ambiguity by suggesting that both isoleucyl-s-RNA and phenylalanyl-s-RNA can compete for the ribosomal binding site when polyU is used as template. Since

liver proteins contain more leucine than phenylalanine, it might be expected that there is more leucyl-s-RNA than phenylalanyl-s-RNA in liver cell sap. If this is so, addition of extra s-RNA would be expected to increase, rather than inhibit, leucine incorporation.

Coding ambiguity towards polyU could be explained in terms of less stringent requirements by the artificial template than by a natural one. But our results suggest that ambiguity can also exist towards a natural RNA template. It seems unlikely that ambiguity occurs *in vivo* and we could argue that ambiguity arises *in vitro* not because of the nature of the template but because the system *in vitro* is less stringent in its requirements than the cell.

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