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Polyribonucleotide Synthesis by Subfractions of Microsomes from Rat Liver

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1. The microsome fraction of rat liver has been fractionated and the ability of the fractions to incorporate ribonucleotides into polyribonucleotides has been studied. Activity was found in the rough-surfaced vesicle (light) fraction and in the free-ribosome fraction and this latter activity has been examined. 2. The free-ribosome fraction contains ribosome monomers, dimers and trimers together with some higher oligomers and ferritin. In addition to catalysing the incorporation of ribonucleotides into acid-insoluble material it contains diesterase activity. It catalyses the incorporation of UMP from UTP, but not UDP, AMP from ATP and CMP from CTP into polyribonucleotide material, and for UTP the product appears to be a homopolymer not more than eight units long attached to the ends of primer polyribonucleotide strands. 3. The activity could not be removed from the free-ribosome fraction by washing or by isolation in the presence of ethylenediaminetetra-acetic acid. 4. Partially hydrolysed polyuridylic acid but not polyadenylic acid could serve as a primer for the incorporation of UMP, but some activity was always associated with an endogenous primer. 5. Analysis of RNA extracted from the free-ribosome fraction after incubation with [^{3}H]UTP showed the presence of 28S, 18S, 5S and transfer RNA types, but no radioactivity was associated with any of these RNA fractions.

We have previously shown (Wilkie & Smellie, 1968) that the cell sap and the microsome fraction of rat liver contain enzymes catalysing the synthesis of polyribonucleotides. The results of experiments with rat liver microsomes suggested the presence of more than one system for the synthesis of polynucleotides and the present studies were undertaken with a view to separating and characterizing the constituent enzyme systems.

EXPERIMENTAL

Nucleotidyltransferase assays, the assay and inactivation of alkaline phosphatase from *Escherichia coli* and the chromatographic and electrophoretic separation and analysis of nucleotides and nucleosides were as described by Wilkie & Smellie (1968).

Subfractionation of rat liver microsomes. The following method was employed to fractionate microsomes into rough-surfaced vesicles (RSV), smooth-surfaced vesicles (SSV) and free ribosomes unattached to membranes. The RSV are believed to derive from fragments of the granular endoplasmic reticulum, i.e. endoplasmic reticulum bearing adherent ribosomes on its outer surface (Moule, Rouiller & Chauveau, 1960); the SSV probably include a variety of membranous forms devoid of ribosomes, such as the agranular endoplasmic reticulum, the plasma membrane and pinocytotic and secretory vesicles (Hallinan & Munro, 1965).

The method is derived from that of Hallinan & Munro (1965). Chilled, diced livers were homogenized in a Sireica homogenizer in 4vol. of 30% sucrose, 0·01M with respect to tris–HCl buffer, pH8·0, 3mM with respect to $MgCl_2$ and 0·5mM with respect to EDTA. About 15 passes were necessary to disrupt the cells, leaving most of the nuclei intact. Particles larger than ribosomes were removed by centrifugation at 18500*g* for 20min. at 0°.

RSV were obtained as a pellet on centrifuging the supernatant at 78000*g* for 60min. at 0°. Two particulate fractions were observed at this stage. A heavier, sticky, opalescent pellet at the bottom of the centrifuge tube was termed the rough-surfaced vesicle heavy (RSVH) fraction. A light fluffy pellet above this was called the rough-surfaced vesicle light (RSVL) fraction. The RSVL fraction suspended very easily in buffer and could be separated from the RSVH fraction. However, it was almost impossible to avoid contamination of the RSVL with the 78000*g* supernatant.

To prepare SSV and free ribosomes, the 78000*g* supernatant fraction was homogenized with 0·5vol. of ice-cold 2,2,4-trimethylpentane (iso-octane) in a Sireica homogenizer until an emulsion had formed (three–four strokes). The emulsion was centrifuged at 150000*g* for 120min. at 0°, when the SSV fraction centrifuged centripetally and formed a pellicle at the interphase between the aqueous phase and

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the iso-octane. Free ribosomes formed a pellet at the bottom of the centrifuge tube.

Particulate fractions were suspended in 0·01 M-tris–HCl buffer, pH 8·0, for enzyme assays.

Extraction of RNA. RNA was extracted from cell extracts or incubation mixtures by a modification of the method described by Penman (1966). The solution containing RNA was poured into 0·1 vol. of washed bentonite suspension in a chilled centrifuge tube. Sodium dodecyl sulphate was added to a concentration of 1% (w/v) and the mixture was allowed to stand for 5·0 min. One volume of 90% (w/v) redistilled phenol in 0·01 M-tris–HCl buffer, pH 7·0, containing 0·1% (w/v) of 8-hydroxyquinoline was added and the mixture was shaken vigorously at room temperature for 5·0 min. One volume of chloroform containing 1% (v/v) of 3-methylbutan-1-ol was added and the mixture shaken vigorously for a further 5·0 min. The resulting emulsion was centrifuged at 1500*g* for 5·0 min., the phenol–chloroform phase was discarded, leaving the interphase material, and the phenol–chloroform extraction was repeated. Extraction of the aqueous phase and interphase was repeated three more times with the chloroform–3-methylbutan-1-ol mixture alone. The aqueous phase was then added to 2 vol. of redistilled ethanol, potassium acetate was added to a concentration of 1% (w/v) and the mixture was allowed to stand at −20° for 1–2 hr. The precipitate of RNA was collected by centrifugation at 15000*g* for 10 min., redissolved and reprecipitated as before. For further manipulation the RNA was dissolved in dilute buffer of the appropriate pH.

Sucrose-gradient analysis. Sedimentation analyses of RNA solutions were performed by centrifugation in 5·0 ml. linear gradients of 5–20% (w/v) sucrose buffered with 0·01 M-potassium acetate, pH 5·2, containing 0·1 M-NaCl and 0·1 mM-$MgCl_2$. Centrifugation was carried out at 0° in the swinging-bucket SW 39 rotor of the Spinco model L ultracentrifuge. The length of time and speed of the runs are indicated in the legends to the appropriate Figures. After centrifugation the bottoms of the tubes were punctured by a syringe needle and 40% sucrose was pumped into them. The contents of the tubes were forced upwards through a Spectronic 505 linear flow spectrophotometer, and $E_{260m\mu}$ was recorded automatically. For radioactivity measurements, the effluents from the Spectronic 505 were collected in five-drop fractions. Nucleic acids were precipitated by the addition of 5·0 ml. of ice-cold 5% (w/v) trichloroacetic acid and 1 mg. of bovine serum albumin. The precipitates were collected by centrifugation at 600*g* at 0°, washed several times in ice-cold 5% (w/v) trichloroacetic acid and prepared for radioactivity measurement as described by Wilkie & Smellie (1968).

Free ribosomes were also analysed as above but sedimentation was through linear 5·0 ml. gradients of 5–20% (w/v) sucrose containing 0·01 M-tris–HCl buffer, pH 8·0, and 2·5 mM-$MgCl_2$.

Preparation of short-chain polyuridylic acid. Polyuridylic acid obtained commercially (K salt) had a sedimentation coefficient greater than 4 S in the analytical ultracentrifuge, and was completely excluded from particles of Sephadex G-100 equilibrated to 0·01 M-tris–HCl buffer, pH 7·0. To distinguish polyuridylic acid from RNA, which was also excluded from the gel particles under these conditions, the molecular size of the polyuridylic acid was reduced by alkaline hydrolysis.

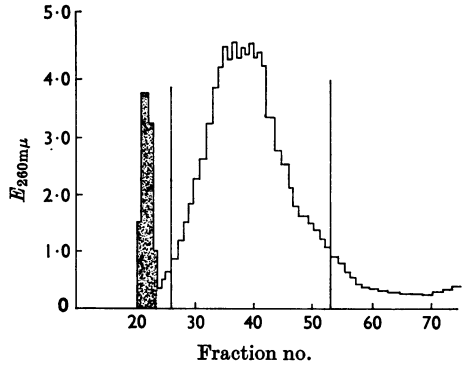

Fig. 1. Chromatography of partially hydrolysed polyuridylic acid on Sephadex G-100. Polyuridylic acid (40 mg.) was hydrolysed in 0·1 N-KOH as described in the Experimental section and applied to a 35 cm. × 2·5 cm. column of Sephadex G-100 equilibrated to 0·01 M-tris–HCl buffer, pH 7·0. The material was eluted with the same buffer and 2·0 ml. fractions were collected and $E_{260m\mu}$ of each was determined. The stippled area gives the elution pattern of the polysaccharide blue dextran, which is completely excluded from the gel particles under these conditions. Material from fractions 27–54 was pooled and the polyuridylic acid was precipitated from 66% (v/v) ethanol containing 1% (w/v) of potassium acetate.

Polyuridylic acid (40 mg.) was hydrolysed in 4·0 ml. of 0·1 M-KOH for 9·0 min. at 37°, the hydrolysate adjusted to pH 7·0 with 1·24 M-$HClO_4$ and insoluble $KClO_4$ removed by centrifugation at 0°. The hydrolysate was then subjected to gel filtration on a column of Sephadex G-100 (35 cm. × 2·7 cm.) equilibrated to 0·01 M-tris–HCl buffer, pH 7·0. The elution pattern of the material is shown in Fig. 1.

Material from the region between fractions 27 and 54 was pooled and the polyuridylate precipitated from 66% ethanol–1% potassium acetate as for RNA. The 200 units of $E_{260m\mu}$ recovered were incubated at 37° for 4·0 min. with sufficient alkaline phosphatase from *E. coli* to release 20 μmoles of *p*-nitrophenol/min. as measured in the alkaline phosphatase assay described by Wilkie & Smellie (1968). The solution was chilled in ice to stop the reaction and the alkaline phosphatase was inactivated as described (Wilkie & Smellie, 1968). The polyuridylate was precipitated from 66% ethanol–1% potassium acetate as before, dissolved in 0·01 M-tris–HCl buffer, pH 7·0, and rechromatographed on Sephadex G-100. Material from fractions 27–54 was pooled, the polyuridylate was precipitated from 66% (v/v) ethanol containing 1% (w/v) of potassium acetate and dissolved in 0·01 M-tris–HCl buffer, pH 8·0. Recovery of 150 units of $E_{260m\mu}$ corresponding to shortened chains of polyuridylate was obtained.

Diesterase activity of cell extracts. The activity of diesterases specific for the 3′-ends of RNA chains was estimated as follows. The cell extract was incubated under normal conditions for the assay of nucleotidyltransferase with the addition of 2 μmoles of *p*-nitrophenyl thymidine 5′-phosphate. The reaction was terminated by cooling the

tubes in crushed ice followed by the addition of 2·5ml. of ice-cold 0·1M-NaOH. The extinction at 400mμ was determined and the amount of *p*-nitrophenol released calculated assuming $E_{max.}$ 12000. All assays were carried out in duplicate with unincubated samples as blanks.

RESULTS

Preliminary experiments showed no incorporation of labelled UMP from UTP by the SSV and RSVH fractions, but activity was observed in the RSVL and free-ribosome fractions. Table 1 shows the effects on these activities of including ATP, GTP and CTP and RNA in the reaction mixtures. The RSVL fraction required the addition of these ribonucleoside 5′-triphosphates before any activity could be demonstrated but the addition of RNA had only a slight effect. The activity of the free-ribosome fraction was slightly reduced by the addition of ATP, GTP and CTP, but RNA had virtually no effect. When the RSVL fraction was resuspended in 0·25M-sucrose containing 2·5mM-magnesium chloride, and centrifuged at 105000*g* for 1hr. at 0°, all the activity appeared in the supernatant fluid. It seems therefore that the activity associated with the RSVL fraction was due to contamination by the 78000*g* supernatant fraction.

Free-ribosome fraction. The specific activity of nucleotidyltransferase in the free-ribosome fraction was often very high and in some experiments between 4 and 5mμmoles of UMP were incorporated/mg. of protein in 5·0min. The reaction showed a linear response to added protein up to a concentration of 2·0mg./ml., and incorporation increased linearly for 10–20min. and showed a maximum between 20 and 40min. (Fig. 2). After this, radioactivity was lost, presumably due to nuclease activity. The reaction was dependent on added magnesium chloride; the Mg^{2+} response curve was practically identical with that obtained

Table 1. *Incorporation of* [^{3}H]*UMP into polynucleotides by subfractions of rat liver microsomes*

Reaction mixtures contained 50μmoles of tris–HCl buffer, pH 8·0, 4μmoles of $MgCl_2$, 0·1μmole of [^{3}H]UTP (20μc/μmole), 2μmoles of phosphocreatine, 20μg. of phosphocreatine kinase and up to 1mg. of protein in a total volume of 0·5ml. Where indicated, 50μg. of highly polymerized yeast RNA and 0·1μmole each of ATP, GTP and CTP were included. Incubations were at 37° for 10min. RSVL, rough-surfaced vesicles (light).

Cell fraction	Additions	UMP incorporated (mμmoles/mg. of protein)
RSVL	—	0·000
	ATP, GTP, CTP	0·410
	RNA	0·045
	RNA, ATP, GTP, CTP	0·396
Free ribosomes	—	1·440
	ATP, GTP, CTP	1·362
	RNA	1·426
	RNA, ATP, GTP, CTP	1·320

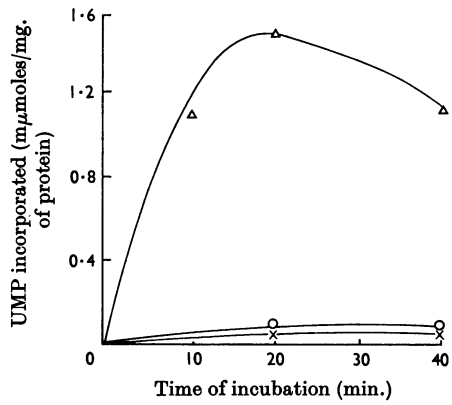

Fig. 2. Utilization of [^{3}H]UTP, [^{3}H]UDP and [^{3}H]UMP by the free-ribosome fraction. Reaction mixtures contained 50μmoles of tris–HCl buffer, pH 8·0, 3μmoles of $MgCl_2$, 10mμmoles of EDTA, 50μg. of highly polymerized yeast RNA and 0·52mg. of protein in a total volume of 0·5ml. As indicated, 0·1μmole of [^{3}H]UTP (△), [^{3}H]UDP (×) or [^{3}H]UMP (○) (20μc/μmole) was included. Incubations were at 37°.

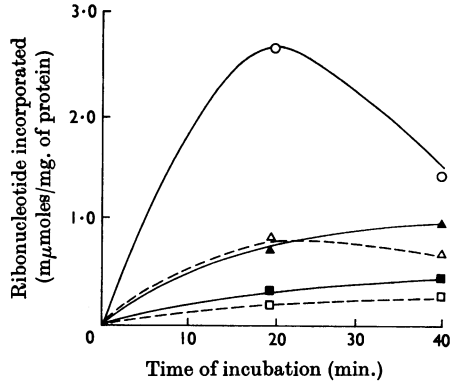

Fig. 3. Utilization of [^{3}H]UTP, [^{3}H]CTP and [^{3}H]ATP by the free-ribosome fraction. Reaction mixtures contained 50μmoles of tris–HCl buffer, pH 8·0, 4μmoles of $MgCl_2$, 10mμmoles of EDTA, 50μg. of RNA and 0·52mg. of protein in a total volume of 0·5ml. Where indicated, 0·1μmole each of [^{3}H]UTP, [^{3}H]CTP or [^{3}H]ATP (20μc/μmole) and 0·1μmole of each of the other three ribonucleoside triphosphates was included. Incubations were at 37°. ○, [^{3}H]UTP; △, [^{3}H]ATP; ▲, [^{3}H]ATP+UTP, CTP+GTP; ■, [^{3}H]CTP; □, [^{3}H]CTP+UTP, ATP+GTP.

for the crude microsome fraction (Wilkie & Smellie, 1968).

The presence of phosphocreatine and phosphocreatine kinase had virtually no effect on the utilization of [^{3}H]UTP. This might be taken to mean that UTP was not the true substrate for the reaction. However, Fig. 2 shows that neither UMP or UDP was utilized in this reaction. The free-ribosome fraction catalysed the incorporation of uracil, adenine and cytosine but not guanine nucleotides, and maximum incorporation was observed at concentrations between 100 and 200mμmoles/reaction tube. Fig. 3 shows the time-course of incorporation of uracil, adenine and cytosine nucleotides into acid-insoluble products. ATP and CTP were utilized to a smaller extent than was UTP. In each case the presence of the three other ribonucleoside 5′-triphosphates inhibited the reaction slightly.

It is known that, in the presence of Mg^{2+}, ribosomes can absorb exogenous enzyme protein from their environment and that treatment of such ribosomes with low Mg^{2+} concentrations or with EDTA can lead to release of such absorbed protein (Tal & Elson, 1961; Neu & Heppel, 1964). Experiments were therefore carried out to test the effects of washing the ribosomes and of preparing them in the presence of high concentrations of EDTA. Table 2 shows that resuspension of the free-ribosome fraction followed by centrifugation in 0·25M-sucrose containing 0·01M-tris–hydrochloric acid buffer, pH 8·0, and 2·5mM-magnesium chloride had virtually no effect on incorporation of uridine nucleotides by this fraction, and suggests that the nucleotidyltransferase activity of the free-ribosome fraction is an integral component and is not due to contamination by the 150000g supernatant fraction.

In another experiment the free ribosomes were prepared by the normal procedure from two samples of the same rat liver. One sample was homogenized in 30% sucrose containing 0·01M-tris–hydrochloric acid buffer, pH 8·0, and 5mM-magnesium chloride, and the other in 30% sucrose–0·01M-tris–hydrochloric acid buffer, pH 8·0, and 12·5mM-EDTA. Free ribosomes were prepared as before and assayed for the ability to incorporate uridine nucleotides into an acid-insoluble product. From Table 3 it can be seen that the preparation of the free ribosomes in EDTA did not diminish their activity but, if anything, enhanced it. This provides further evidence that the nucleotidyltransferase activity is not absorbed from the soluble fraction by a fortuitous mechanism, but either is bound to the ribosome structure or is large enough to sediment with the ribosomes.

Fig. 4(*a*) shows the extinction profile at 260mμ obtained on analysis of the free ribosomes in a sucrose gradient. Five distinct peaks were observed. Measurements were also made of the extinction at 320mμ to determine whether ferritin was present in the free ribosome fraction (Munro, Jackson & Korner, 1964). Fig. 4(*b*) shows that a single peak, which coincided with peak II, was observed. The total extinction of the peak in Fig. 4(*b*) at 260mμ was estimated by applying the correction factor 1·5, used by Munro *et al.* (1964), and it was found to represent 90% of the material in peak II of Fig. 4(*a*). It was therefore concluded that peak II is ferritin and that peak III corresponded to ribosome monomers, peak IV to ribosome dimers and peak V to ribosome trimers. The small peak sedimenting near the top of the gradient probably represents degraded RNA and protein. Thus the free ribosomes that have been used in our experiments consist of a mixture of ribosome monomers, dimers and trimers, together with smaller amounts of oligomeric ribosomes, and a substantial amount of ferritin.

When the free-ribosome fraction was isolated in 0·01M-EDTA the profiles obtained on analysis in sucrose gradients closely resembled that in Fig. 4(*a*) but the relative proportion of ferritin was reduced.

Table 2. *Effect of washing the free-ribosome fraction on the incorporation of* [^{3}H]*UMP into polyribonucleotides*

Reaction mixtures contained 50μmoles of tris–HCl buffer, pH 8·0, 4μmoles of $MgCl_2$, 0·1μmole of [^{3}H]UTP (20μc/μmole), 10mμmoles of EDTA and 0·53mg. of protein in a total volume of 0·5ml. Incubations were at 37° for 5·0min. Free ribosomes were prepared as described in the Experimental section. One portion of the free-ribosome fraction was assayed without further treatment and a second portion was resuspended in 0·25M-sucrose–0·01M-tris–HCl buffer, pH 8·0–2·5mM-$MgCl_2$ and centrifuged at 150000g for 2hr. before assay.

Treatment	UMP incorporated (mμmoles/mg. of protein)
Unwashed	4·11
Washed	4·41

Table 3. *Effect of isolating the free-ribosome fraction in a medium containing* 0·01M-*EDTA on the incorporation of* [^{3}H]*UMP into polyribonucleotides*

Reaction mixtures were as given in Table 2. There was 0·8mg. of enzyme protein per tube. Incubations were at 37° for 5·0min.

Isolation medium	UMP incorporated (mμmole/mg. of protein)
Mg^{2+}	0·750
EDTA	0·950

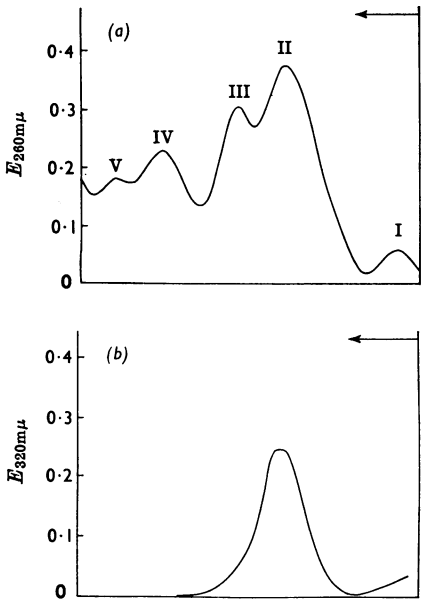

Fig. 4. Sucrose-gradient analysis of the free-ribosome fraction from rat liver cells. The free-ribosome fraction was prepared from the liver of one animal by the normal method. The 150000*g* pellet was suspended in 0·45ml. of 0·01M-tris–HCl buffer, pH7·0, 2·5mM with respect to $MgCl_2$. A portion (0·06ml.) of this suspension was layered on top of 5·0ml. of 5–20% sucrose gradient buffered with 0·01M-tris–HCl, pH7·0, containing 2·5mM-$MgCl_2$. The gradient was centrifuged in the SW 39 head of the MSE 50 centrifuge at 38000rev./min. for 1hr. 30min. The extinction of the gradient was measured (*a*) at 260mμ, (*b*) at 320mμ.

This may account for the higher activity observed for ribosomes prepared from EDTA and suggests that the nucleotidyltransferase activity is a function of the ribosomes rather than of ferritin.

The observation that UMP incorporation is not stimulated by the presence of ATP, GTP and CTP suggests that the product of the reaction is a homopolymer. This is borne out by the results presented in Tables 4 and 5. Table 4 shows the distribution of ^{32}P in the ribonucleoside 3′(2′)-monophosphates obtained on alkaline hydrolysis of the product of the reaction in an experiment with [α-^{32}P]UTP (Wilkie & Smellie, 1968). When [α-^{32}P]UTP was the only ribonucleotide in the reaction mixture more than 90% of the ^{32}P was recovered in 3′(2′)-UMP. The addition of ATP, GTP and CTP to reaction mixtures caused a slight fall in the proportion of ^{32}P recovered in 3′(2′)-UMP, but even in these circumstances the proportion was nearly 80%. The average length of the newly incorporated uridylate sequences was also determined as described by Wilkie & Smellie (1968).

Table 4. *Distribution of ^{32}P in the ribonucleoside 3′(2′)-monophosphates obtained on alkaline hydrolysis of the product obtained after incorporation of [α-^{32}P]UMP by the free-ribosome fraction*

Reaction mixtures contained 200μmoles of tris–HCl buffer, pH8·0, 12μmoles of $MgCl_2$, 40mμmoles of EDTA, 0·4μmole of [α-^{32}P]UTP (20×10^6counts/min./μmole), 200μg. of RNA and 1·9mg. of protein in a total volume of 2·0ml. Incubations were at 37°. Where indicated, 0·1μmole each of ATP, GTP and CTP was included.

Incubation time and additions	Recovery of ^{32}P (%) In UMP	In CMP	In AMP	In GMP
10min.	92	2·3	2·7	3·0
40min.	92·5	1·8	2·4	3·3
10min. (+ATP+GTP+CTP)	78	10·9	5·4	5·7
40min. (+ATP+GTP+CTP)	77·4	11·6	5·8	5·2

Table 5. *Average chain length of the product formed after incubation of the free-ribosome fraction with [^{3}H]UTP*

Reaction mixtures contained 50μmoles of tris–HCl buffer, pH8·0, 4μmoles of $MgCl_2$, 10mμmoles of EDTA, 0·1μmole of [^{3}H]UTP (200μc/μmole) and 0·344mg. of protein in a total volume of 0·5ml. Incubation was at 37° for 40min.

Additions	Ratio of radioactivity / nucleotide / nucleoside	Average chain length
—	7·10	8
ATP, GTP, CTP	3·90	5

In the absence of ATP, GTP and CTP the value obtained was 8 and in the presence of these ribonucleotides it was 5. This slightly lower value may represent the formation of shorter homopolymer chains when the other ribonucleotides are present or a very small degree of heteropolymer formation.

In view of the relatively short chains formed in this system and the relatively high rates of incorporation of uridine nucleotides observed, it seemed possible that the polynucleotide product was being degraded by nucleolytic enzymes in the free-ribosome fraction. Measurements of phosphodiesterase activity in this fraction were therefore carried out with *p*-nitrophenyl thymidine 5′-phosphate as a substrate. This provides a measure of the activity of diesterases attacking polynucleotide chains from

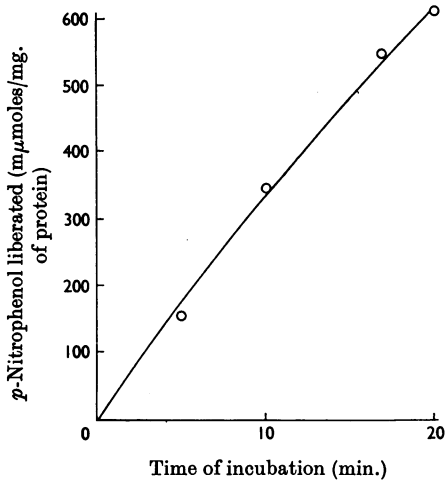

Fig. 5. 3′-Phosphodiesterase activity of the free-ribosome fraction. Reaction mixtures contained 50 μmoles of tris–HCl buffer, pH 8·0, 4 μmoles of $MgCl_2$, 10 mμmoles of EDTA, 0·1 μmole of UTP, 2 μmoles of *p*-nitrophenyl thymidine 5′-phosphate and 0·252 mg. of protein in a total volume of 0·5 ml. Incubations were at 37°. The reaction was terminated by cooling the tubes in crushed ice.

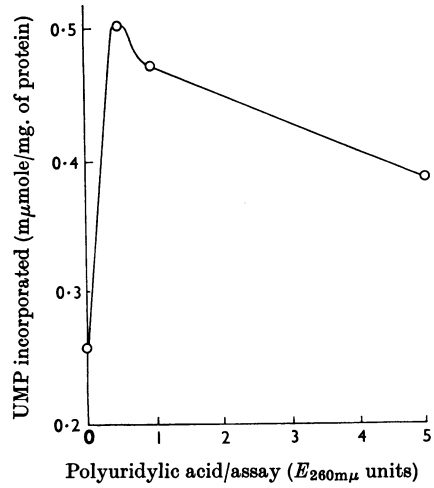

Fig. 6. Effect of increasing concentrations of partially hydrolysed polyuridylic acid on the incorporation of [^{3}H]UMP by the free-ribosome fraction. Reaction mixtures contained 50 μmoles of tris–HCl buffer, pH 8·0, 4 μmoles of $MgCl_2$, 10 mμmoles of EDTA, 0·1 μmole of [^{3}H]UTP (20 μc/μmole) and 0·465 mg. of protein in a total volume of 0·5 ml. Various amounts of partially hydrolysed polyuridylic acid were included as shown. Incubations were at 37° for 5·0 min.

the 3′-end. The results of these experiments are shown in Fig. 5. High activities for diesterase were observed, and it is therefore possible that longer chain lengths are synthesized *in vitro*. This result also helps to account for the loss of radioactivity from the reaction product when incubations are carried on beyond the optimum time.

All the results described above are consistent with a reaction leading to the extension of existing polynucleotide chains. Further proof of this was obtained from experiments with partially hydrolysed polyuridylic acid primers prepared as already described. Reaction mixtures were prepared with the free-ribosome fraction, and varying amounts of partially hydrolysed polyuridylate primer were added. Fig. 6 shows that addition of this primer led to increased incorporation up to a value of 1 $E_{260m\mu}$ unit per assay. In further experiments with the same free-ribosome preparation in the presence and absence of added polyuridylate, polyribonucleotides were extracted from the reaction mixtures after incubation and analysed on columns of Sephadex G-100 as already described. The results of these experiments are shown in Fig. 7. In the absence of polyuridylate primer there is one main peak of extinction and the radioactivity corresponds closely to this. The added polyuridylate is clearly well separated from the endogenous RNA of the free ribosomes and in this experiment radioactivity occurs both in the endogenous RNA and in the polyuridylate. Of the radioactivity 48% was recovered in endogenous RNA and 52% in polyuridylate and this agrees well with the calculation made from the results in Fig. 6.

The possibility remained that the uridine nucleotides associated with the endogenous primer were not attached by covalent linkage but merely by weaker forces such as hydrogen bonding to complementary residues in the primer strand. Accordingly, the ability of polyuridylate and polyadenylate to support the incorporation of adenine and uracil nucleotides respectively was examined. From Fig. 8 it can be seen that although polyuridylate acted as an efficient primer for uptake of uracil nucleotides, polyadenylate inhibited the reaction. Conversely, although polyadenylate was a rather poor primer for incorporation of adenine nucleotides, polyuridylate inhibited this reaction. From the results, it is clear that a base-pairing mechanism is not involved in the reaction.

Other experiments have been performed to investigate which molecular species of RNA from the free-ribosome fraction served as a primer for incorporation of ribonucleotides. The free-ribosome fraction was incubated in the usual way with [^{3}H]UTP and RNA was extracted from it with phenol as before. The isolated RNA was analysed on sucrose density gradients and the results are

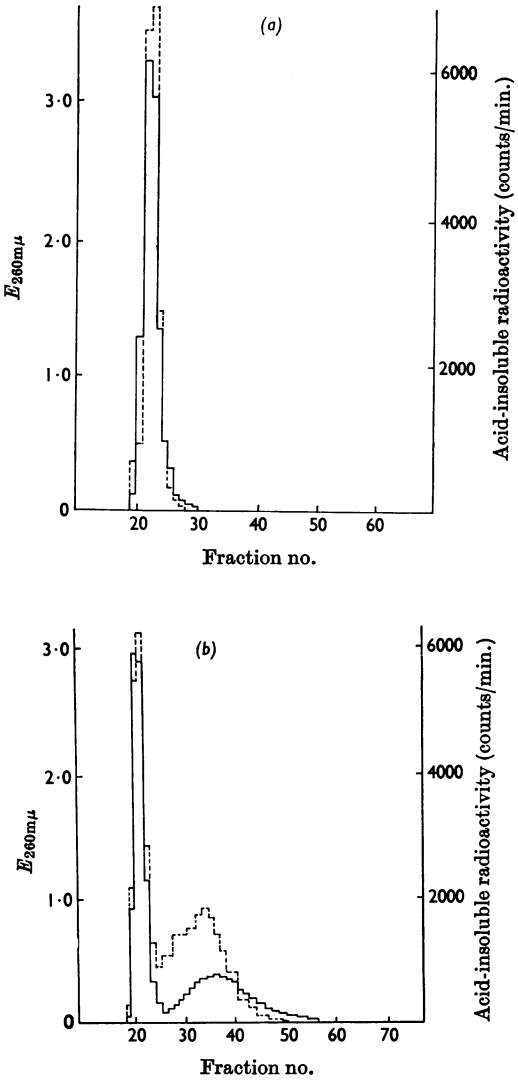

Fig. 7. Fractionation on Sephadex G-100 of the product obtained on incubating the free-ribosome fraction with [^{3}H]UTP. (*a*) Reaction mixture contained 750μmoles of tris–HCl buffer, pH 8·0, 60μmoles of $MgCl_2$, 0·15μmole of EDTA, 1·5μmoles of [^{3}H]UTP (66·6μc/μmole) and 7·0mg. of protein in a total volume of 7·5ml. Incubation was at 37° for 5·0min. The isolated RNA was dissolved in 0·01M-tris–HCl buffer, pH 7·0, and fractionated on a column of Sephadex G-100 (35cm.×2·5cm.) Fractions (2ml.) were collected and $E_{260m\mu}$ was measured. The RNA in each fraction was then precipitated by the addition of 5·0ml. of ice-cold 5% (w/v) trichloroacetic acid and the precipitate was collected by centrifugation. The precipitate was washed three times with 5% (w/v) trichloroacetic acid and radioactivity was measured in a scintillation spectrometer. (*b*) As for (*a*) but 15 $E_{260m\mu}$ units of partially hydrolysed polyuridylic acid were added. ———, $E_{260m\mu}$; ·····, acid-insoluble radioactivity (counts/min.).

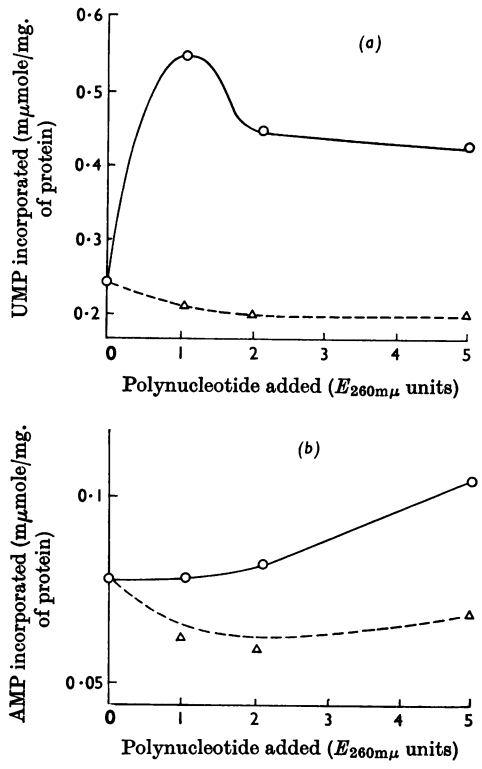

Fig. 8. Effect of increasing amounts of polyadenylic acid and polyuridylic acid on the utilization of [^{3}H]UTP and [^{3}H]ATP by the free ribosome fraction. (*a*) Reaction mixtures contained 50μmoles of tris–HCl buffer, pH 8·0, 4μmoles of $MgCl_2$, 10mμmoles of EDTA, 0·1μmole of [^{3}H]UTP (20μc/μmole) and 0·494mg. of protein in a total volume of 0·5ml. Where indicated, polyadenylic acid or polyuridylic acid was included. Incubation was at 37° for 5·0min. ○, Polyuridylic acid; △, polyadenylic acid. (*b*) As for (*a*) but [^{3}H]UTP was replaced by [^{3}H]ATP. ○, Polyadenylic acid; △, polyuridylic acid.

shown in Fig. 9. Three distinct peaks of extinction at 260mμ were observed. Two of these appeared to correspond to the two large ribosomal species (28s and 18s) whereas the third, lighter, component was located close to the top of the gradient. Some at least of this component seemed likely to be degraded 28s or 18s RNA. No significant amount of radioactivity was present in the 28s or 18s components and nearly all of it was associated with the slowly sedimenting material. Unincubated samples gave a similar distribution of ultraviolet-absorbing material, but no radioactivity was found in any fraction.

The results suggested that either t-RNA (transfer RNA), which is known to be attached to the ribosomes (Galibert, Larson, Lelong & Boiron, 1965), or

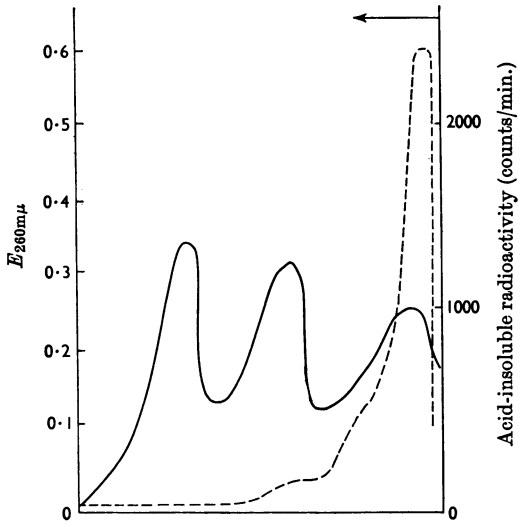

Fig. 9. Sucrose-density-gradient analysis of RNA isolated from free ribosomes after incubation with [^{3}H]UTP. Reaction mixtures were prepared containing 1·5m-moles of tris–HCl buffer, pH 8·0, 120μmoles of $MgCl_2$, 0·3μmole of EDTA, 3μmoles of [^{3}H]UTP (33·3μc/μmole) and 21·6mg. of protein of the free-ribosome fraction in a total volume of 15ml. Incubations were for 5min. at 37°. RNA was extracted with phenol and dissolved in 0·01M-potassium acetate, pH 5·2–0·1M-NaCl–0·4mM-$MgCl_2$. A portion (0·04ml.) of this RNA solution (containing 2·44 $E_{260m\mu}$ units) was layered on top of a 5–20% sucrose gradient containing the same buffer. The gradient was centrifuged at 39000rev./min. in the SW 39 rotor of the Spinco model L ultracentrifuge for 3½hr. at 2°. The extinction profile at 260mμ of the gradient was measured by the continuous-flow method, and the eluate was collected in 28 fractions. A portion (0·5mg.) of bovine serum albumin was added to each fraction followed by 5·0ml. of ice-cold 5% (w/v) trichloroacetic acid. Acid-precipitable material was washed four times in trichloroacetic acid and the radioactivity measured. ——, $E_{260m\mu}$; ·····, acid-insoluble radioactivity (counts/min.).

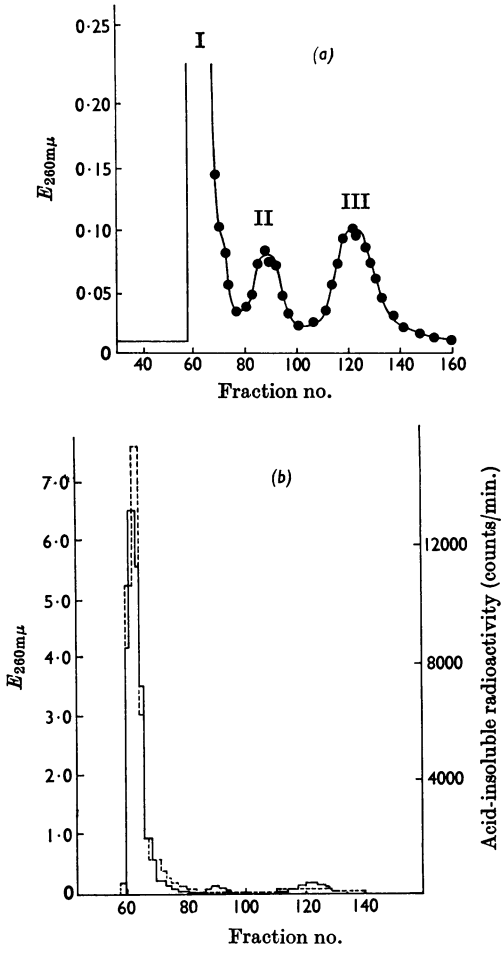

Fig. 10. Fractionation of RNA from the free-ribosome fraction on Sephadex G-100. Free ribosomes were incubated for 5·0min. at 37° in a reaction mixture containing 2·5m-moles of tris–HCl buffer, pH 8·0, 200μmoles of $MgCl_2$, 0·5μmole of EDTA, 5μmoles of [^{3}H]UTP (a total of 200μc) and 25·6mg. of protein in a total volume of 25ml. The RNA was then extracted as before and applied to a column of Sephadex G-100 (94cm. × 2·5cm.), which had been equilibrated at 1–3° with 0·05M-potassium acetate, pH 5·1. Elution was with the same buffer and 2·0ml. fractions were collected. $E_{260m\mu}$ and the acid-insoluble radioactivity were determined as before. The full extinction profile and radioactivity are given in (*b*). (*a*) The extinction profile shown on a sufficiently large scale to demonstrate peaks I, II and III. (*b*) The extinction profile with the radioactivity superimposed on it. ——, $E_{260m\mu}$; ------, acid-insoluble radioactivity (counts/min.).

5s ribosomal RNA or degradation products of ribosomal RNA were acting as primers. The method of Galibert *et al.* (1965) was employed to separate t-RNA and 5s RNA from the bulk of the free-ribosomal RNA. A column of Sephadex G-100 (94cm. × 2·5cm.) was equilibrated with 0·05M-potassium acetate buffer, pH 5·1. To minimize degradation of RNA the column was prepared and used with a water jacket cooled to between 1° and 3°. The free-ribosome fraction was incubated for 5min. with [^{3}H]UTP under the usual conditions, RNA was extracted with phenol, applied to the Sephadex column and eluted with ice-cold potassium acetate, pH 5·1. Fig. 10(*a*) illustrates the elution patterns obtained. t-RNA extracted from the 150000*g* supernatant fraction coincided with peak III and it was concluded that this peak represented t-RNA and that peak II corresponded to 5s ribosomal

RNA. Virtually all the radioactivity was associated with peak I and it seems unlikely therefore that this t-RNA or 5s RNA species act as primers in the system.

DISCUSSION

Only two of the subfractions obtained from rat liver microsomes were active in incorporating uracil nucleotides into acid-insoluble material. These were the RSVL fraction and the free-ribosome fraction, and the characteristics of these two systems were very different. The reaction catalysed by the RSVL fraction required supplementation by ATP, GTP and CTP and showed some response to the addition of RNA. The free-ribosome fraction, however, was little affected by supplementation with either ATP, GTP and CTP or RNA.

Although the RSVL fraction was of particular interest since its activity showed some of the characteristics of that in the whole-microsome fraction (Wilkie & Smellie, 1968) it was likely to be contaminated and, in view of this and of the loss of activity after washing, no further studies were carried out with it.

The specific activity of the free-ribosome fraction with respect to the incorporation of uracil nucleotides was relatively high and in line with other observations on similar systems (Burdon, 1963; Wykes & Smellie, 1966). However, the reaction seems to represent the formation of homopolymers containing not more than eight uridylate residues attached to the ends of endogenous RNA primers. It is possible that longer polymers are synthesized but that they are degraded by nucleases in the system since we have observed quite high diesterase activity in the free ribosome preparations (Fig. 5).

Attempts to determine which molecular species of RNA serve as primers for this reaction have enabled us to exclude t-RNA and 5s RNA, neither of which became labelled in our experiments. Equally ribosomal RNA, as such, does not become labelled although degradation products of ribosomal RNA may serve as primers in this system. It seems a little surprising, however, that the enzyme responsible for incorporating uracil nucleotides should be able to distinguish rather effectively between ribosomal RNA and oligonucleotides derived from ribosomal RNA.

The only other species of RNA known to be present in this system is messenger RNA and it may be that enzymes exist in free ribosomes having the capacity to modify messenger RNA molecules by the addition of terminal homopolymer sequences. We have no direct evidence, however, to implicate messenger RNA as the nucleotide acceptor in this system.

The biological significance of the reactions described in this paper is far from clear. There exists, in the free-ribosome fraction of rat liver, a relatively active system for the addition of ribonucleotide residues to polyribonucleotide chains, and similar enzymic activities have been observed in the microsome fraction from pigeon liver (Straus & Goldwasser, 1961), Krebs 2 mouse ascites-tumour cells (Cline, Eason & Smellie, 1963; Horton, Liu, Martin & Work, 1966) and Landschütz ascites-tumour cells (Wykes & Smellie, 1966). The reactions do not require DNA or the complete set of ribonucleoside 5′-triphosphates and cannot therefore be attributed to DNA-dependent RNA-polymerase. It is possible that sub-units of this enzyme, perhaps newly synthesized, are associated with ribosomes and could exhibit the type of activity we have observed, but we think that the relatively high specific activity of the free-ribosome fraction argues against this conclusion.

In the ribosome fraction of *E. coli* an enzyme has been demonstrated that is capable of adding up to 200 adenylate residues to added RNA primers (August, Ortiz & Hurwitz, 1963; Smith & August, 1966) and there have been reports of the occurrence of polyadenylic acid in rat liver microsomes (Hadjivassiliou & Brawerman, 1966). The free-ribosome fraction of rat liver can form polyadenylate but, unlike the ribosomes from *E. coli*, it is more active in catalysing the synthesis of uridylate polymers. So far there has been no report of polyuridylate occurring in rat liver microsomes.

At the present time we have no evidence from which to propose any function for the activities observed in the free-ribosome fraction. They may only be artifacts brought about by the conditions of assay or of isolation of the free-ribosome fraction, although the relatively high activity tends to argue against this. Alternatively they may be involved in the modification of RNA species within the cell so as to suit them to their physiological role.

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