The Synthesis of Polyrribonucleotides by Cytoplasmic Enzymes

BY J. R. WYKES AND R. M. S. SMELLIE

Department of Biochemistry, University of Glasgow, Scotland

(Received 26 October 1965)

1. The possibility that the cell cytoplasm contains enzymes catalysing the biosynthesis of RNA was investigated in fractions obtained by differential centrifugation of homogenates of Landschutz ascites-tumour cells. 2. The microsomal fraction was shown to be most active in incorporating UMP residues from [α - 32 P]UTP into polyribonucleotide material. 3. The same fraction also incorporated [3 H]CTP, [3 H]ATP and [3 H]GTP separately and independently of the presence of complementary ribonucleoside 5'-triphosphates. 4. The reaction was promoted by the addition of RNA and showed an absolute requirement for Mg^{2+} ions. 5. Analysis of alkaline hydrolysates of the reaction products after the incorporation of [α - 32 P]UTP showed that most of the radioactivity was recovered in (2',3')-UMP residues irrespective of whether CTP, ATP and GTP were present in the reaction mixture. 6. Extraction of RNA from the reaction mixtures after the incorporation of [3 H]ATP, [3 H]GTP or [3 H]CTP and analysis by sucrose-density-gradient centrifugation showed no labelling of the ribosomal RNA. Radioactive material appeared between the 4s region and the meniscus of the sucrose gradient. In agreement with this observation, determinations of the chain length of the product showed that only short sequences of polynucleotides were synthesized. It is concluded that only homopolyribonucleotide synthesis is catalysed by the microsomal fractions and that there is little or no synthesis of RNA-like heteropolymers.

Evidence from studies of the kinetics of incorporation of labelled nucleosides into RNA, the effect of actinomycin D on such incorporation (Perry, Hell & Errera, 1961; Perry, 1962; Scherrer, Latham & Darnell, 1963) and the ability of the major cellular RNA components to form hybrids with homologous DNA (Yanofsky & Spiegelman, 1962, 1963; Goodman & Rich, 1962) indicate that virtually all synthesis of RNA is carried out on a DNA template. Further, DNA-primed RNA-polymerase activity has been detected in association with chromatin material (Huang & Bonner, 1962) and a similar enzyme has been demonstrated in several animal tissues (Weiss, 1960; Eason & Smellie, 1965). Nevertheless, several systems of cytoplasmic origin are known which incorporate ribonucleotides into polyribonucleotides. Straus & Goldwasser (1961) detected incorporation of UMP residues into a microsomal fraction from pigeon liver. Cytoplasmic extracts from Landschutz ascites-tumour cells have also been found to incorporate UMP residues into polyribonucleotide material (Burdon & Smellie, 1961, 1962). A similar system that incorporates UMP residues into polyuridylylate and AMP residues into polyadenylylate has been partially purified from rat liver (Klemperer, 1963*a,b*).

The present paper is concerned with the further investigation and characterization of the system described by Burdon & Smellie (1961, 1962) in ascites-tumour cells with a view to establishing whether or not it is concerned with the synthesis of RNA in the cytoplasm.

EXPERIMENTAL

Materials. [3 H]Ribonucleoside 5'-triphosphates and [3 H]deoxyadenosine 5'-triphosphate were obtained from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A. Carrier-free [32 P]orthophosphate and cyanoethyl [32 P]phosphate were obtained from The Radiochemical Centre, Amersham, Bucks. Thymidine 5'-[α - 32 P]triphosphate was a gift from Dr J. B. Shepherd. *p*-Nitrophenyl thymidine 5'-phosphate was obtained from Calbiochem, Los Angeles, Calif., U.S.A., and *p*-nitrophenyl thymidine 3'-phosphate was a gift from Dr P. J. Curtis.

Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Purified yeast *s*-RNA was a gift from Dr D. Bell. [32 P]UMP was prepared from [32 P]orthophosphate or cyanoethyl [32 P]phosphate by the method of Tener (1961) and phosphorylated by the method of Smith & Khorana (1958) to yield [α - 32 P]UTP.

Preparation of extracts. Landschutz ascites carcinoma was maintained by serial transplantation in Porton strain white mice of the departmental colony. Tumour was withdrawn from the mice after 7 days and collected in ice-cooled graduated centrifuge tubes. The ascites-tumour

cells were collected by centrifuging the tumour suspensions at 600g for 10 min. in an MSE refrigerated centrifuge. All further operations were performed at 1–3° in ice-cooled apparatus. The cells were broken by a modification of the procedure of Fisher & Harris (1962) in 5 vol. of a medium containing 0.01 M-tris-HCl buffer, pH 8.0, 0.5 mM-MgCl₂ and 0.1% (v/v) Tween 80 by repeated passage through a Potter-type homogenizer until microscopic examination of smears stained with crystal violet showed that at least 95% of the cells were broken and clumping of nuclei was observed. Nuclei and mitochondria were removed by centrifugation of the cell homogenates at 12000g for 20 min. If the mitochondria were required nuclei were first removed by centrifugation at 600g for 20 min. and the supernatant fraction was then centrifuged again at 12000g for 20 min. to yield a pellet of mitochondria. The pellets so obtained were washed by suspension in a small volume of 0.01 M-tris-HCl buffer, pH 8.0, and centrifuged again at 12000g. Microsomes were prepared by centrifuging the 12000g supernatant fraction at 105000g for 2 hr. in a Spinco model L or MSE Superspeed 50 centrifuge. In some experiments the 105000g supernatant fraction was centrifuged again at 150000g for 2 hr. to give a pellet and a 150000g supernatant fraction. The pellets obtained by centrifugation were resuspended by gentle homogenization in 10 mM-tris-HCl buffer, pH 8.0, to give a protein concentration of 2–3 mg./ml.

In some cases an acetone-dried powder of the microsomes was prepared. The microsomal pellet was homogenized in a small volume of 10 mM-tris-HCl buffer, pH 8.0, to give a thick suspension which just flowed on pouring. Usually the microsomal pellet from 200 ml. of disrupted cell homogenate was homogenized in 3 ml. of buffer. A portion (1 ml.) of the microsomal suspension was poured into 80 ml. of acetone cooled in a solid CO₂-ethanol freezing mixture. The suspension was rapidly dispersed in an MSE Nelco blender at full speed for 1.5 min. Insoluble material was collected by centrifugation at 600g for 5 min. at –15° to –20° and washed three times by resuspension in 80–100 ml. of acetone at the same temperature. Finally, the sediment was dried under vacuum, giving a white powder which was stored at –15°. For assay purposes this powder was suspended in 10 mM-tris-HCl buffer, pH 8.0.

Assay procedure. After incubation the reaction mixtures were processed by a procedure based on the method of Baltimore & Franklin (1963). All operations were performed at 1–3°. A portion (1 mg.) of carrier protein (0.5 ml. of 0.2% bovine serum albumin) was added to the reaction mixture, followed by 5 ml. of 5% (w/v) trichloroacetic acid containing 10% (v/v) saturated Na₄P₂O₇ neutralized to pH 5–6 with H₃PO₄ (solution A), and the precipitate was collected by centrifugation at 600g for 10 min. The sediment was dissolved in 0.5 ml. of 0.5 N-NaOH and immediately reprecipitated with 5 ml. of solution A. Subsequent washing was performed by suspending the sediment in 1 ml. of solution A with a Vortex mixer (Scientific Instruments Ltd.) and the addition of a further 4 ml. of solution A and allowing to equilibrate in an ice bath for 10 min. before centrifuging again. This washing procedure was performed five times and RNA was hydrolysed with 0.6 ml. of 5% trichloroacetic acid by heating at 95–100° for 20 min. in a water bath. Insoluble material was removed by centrifugation and samples of the clear supernatant fluid were transferred to vials for measurements of radioactivity. In some experiments with [α -³²P]UTP, the washed sediment was

finally dissolved in 1 ml. of concentrated formic acid, transferred to stainless-steel planchets and dried under infrared lamps.

³H was measured in a three-channel Nuclear-Chicago model 725 liquid-scintillation spectrometer with a scintillator consisting of Scinstant NE 572 (Nuclear Enterprises, Edinburgh) dissolved in dioxan. ³²P was measured with a Nuclear-Chicago gas-flow counter fitted with a Micromil window.

Preparation of RNA. RNA from whole cell ascites-tumour cells, for use as a primer in reaction mixtures, was prepared by the method of Eason, Cline & Smellie (1963). Analysis by sucrose-density-gradient centrifugation showed the RNA to be of high molecular weight, containing the characteristic 30s, 18s and 4s peaks.

Extraction of RNA from reaction mixtures. RNA was extracted from reaction mixtures (containing a sufficient amount of microsomal material to give 1 mg. of RNA/assay tube) by the following procedure. A portion (0.5 ml.) of 0.1 M-sodium acetate buffer, pH 5.2, 0.05 ml. of 0.5% (w/v) sodium dodecyl sulphate and 0.05 ml. of 0.1% (w/v) bentonite suspension were added to 0.5 ml. of reaction mixtures. The reagents were mixed in a Vortex mixer and shaken with 0.7 ml. of 90% (w/v) phenol for 10 min. at room temperature. The resulting emulsion was broken by centrifugation at 10000g for 5 min. and the aqueous layer was transferred to 15 ml. centrifuge tubes containing 50 μ g. of bentonite. The interphase residue was re-extracted twice more by adding 0.7 ml. of 0.01 M-sodium acetate buffer, pH 5.2, shaking for 10 min. and centrifuging at 10000g for 5 min. The combined aqueous fractions were extracted with 1 ml. of ether and RNA was precipitated with 2 vol. of ethanol containing 1% (w/v) of potassium acetate. After cooling at –15° for 30 min. the precipitate was collected by the centrifugation at 600g for 30 min. The sediment was washed twice by dissolving in 0.01 M-sodium acetate, 0.05 M-NaCl and 0.5 mM-MgCl₂ adjusted to pH 5.2 (solution B) and reprecipitating with ethanol-potassium acetate as before. After the initial precipitation, RNA from duplicate tubes was usually combined. The RNA was dissolved in 2 ml. of solution B and dialysed against two changes of 7 l. of the same buffer for 18 hr. The dialysed solution was clarified by centrifugation at 600g for 10 min. and the RNA was reprecipitated from the supernatant as before. The final precipitate was dissolved in 0.4 ml. of solution B and extracted twice with an equal volume of ether. The last traces of ether were removed by a stream of air. The solutions which contained 0.5–0.75 mg. of RNA were analysed by sucrose-density-gradient centrifugation (Britten & Roberts, 1960).

Nuclease determination. Nuclease activity was determined using the same reaction conditions as for nucleotide incorporation except that ribonucleoside 5'-triphosphates were omitted. When RNA was used as the substrate the reaction was stopped with 3.5 ml. of 1 N-HClO₄, the precipitate was removed by centrifuging at 600g for 10 min. and the extinction of the supernatant fluid was measured at 260 m μ . The amount of nucleotide rendered acid-soluble was calculated assuming E_{max} 10600. When *p*-nitrophenyl thymidine 5'-phosphate or *p*-nitrophenyl thymidine 3'-phosphate was used as substrate the reaction was stopped with 3.5 ml. of 0.1 N-NaOH, any insoluble material was removed by centrifuging at 600g for 10 min. and the extinction of the supernatant fluid was measured at 400 m μ .

The amount of nucleotides hydrolysed was calculated assuming E_{max} 12000.

Protein estimation. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Fig. 1 shows the time-course of incorporation of [α - 32 P]UMP residues into polyribonucleotides by a mitochondrial fraction, a 105 000g sediment and a 105 000g supernatant fraction of Landschutz ascites-tumour cells. Only the 105 000g sediment (microsomal fraction) shows appreciable activity. The supernatant fraction shows low activity and the mitochondrial fraction negligible activity. The 105 000g supernatant fraction was further fractionated by centrifugation at 150 000g for 2 hr., giving a sediment and a supernatant fraction. The incorporation of these fractions, a microsomal fraction and a 105 000g supernatant fraction, is compared in Fig. 2. Again the microsomal fraction shows most activity although some activity is recovered in the 150 000g sediment and the supernatant fractions show low activity. The UMP-incorporating activity thus appears to be associated with microsomal material. This conclusion gained support from experiments in which the microsomal

fraction was centrifuged on sucrose-density gradients. Fig. 3a shows the distribution of extinction at 260m μ and 280m μ of a microsomal fraction sedimented on a sucrose-density gradient together with measurements of the ability to incorporate [3 H]UTP. The peak of incorporation is displaced to the lighter side of the extinction peak. Attempts were made to dissociate the microsomal fraction by dialysis against 10mm-tris-hydrochloric acid buffer, pH 8.0. The extinction profile obtained on sucrose-density-gradient centrifugation (Fig. 3b) shows some dissociation of the microsomes into lighter fractions although the incorporating activity still remains associated with particulate material.

The properties of the reaction were further investigated with an acetone-dried powder prepared from the microsomal fraction. The acetone-dried powder retained 80–90% of the activity of the microsomal fraction from which it was prepared and could be stored for up to 2 weeks at -15° without further loss of activity. Preparation of an acetone-dried powder from the microsomal fraction did not alter the characteristics of ribonucleotide incorporation. The acetone-dried powder was found to incorporate [3 H]ATP, [3 H]GTP, [3 H]CTP and [3 H]UTP into acid-precipitable material to somewhat different extents under the same reaction conditions. Optimum incorporation of labelled ribonucleotide was obtained in appropriately buffered reaction mixtures containing a single labelled ribonucleoside 5'-triphosphate, RNA, creatine phosphate and creatine kinase (EC 2.7.3.2), and Mg^{2+} ions. EDTA and 2-mercaptoethanol were always included in the reaction mixture as these reagents stimulated the ribonucleotide incorporation. Table 1 shows the effects

Fig. 1. Time-course of the incorporation of [α - 32 P]UTP by subcellular fractions of Landschutz ascites-tumour cells. The reaction mixture contained: 50 μ moles of tris-HCl buffer, pH 8.5; 5 μ moles each of MgCl_2 and 2-mercaptoethanol; 0.5 μ mole of EDTA; 0.1 μ mole of [α - 32 P]UTP (4.5×10^6 counts/min./ μ mole); 2 μ moles of phosphocreatine; 50 μ g. of phosphocreatine kinase; 100 μ g. of RNA; 0.75 mg. of protein; total volume, 0.5 ml. Incubations were performed at 37° for the times indicated. ▲, Microsomal fraction; ○, 105 000g supernatant fraction; ×, mitochondrial fraction.

Fig. 2. Time-course of the incorporation of [α - 32 P]UTP by subcellular fractions of Landschutz ascites-tumour cells. The reaction mixture and conditions were as described for Fig. 1. ▲, Microsomal fraction; ■, 150 000g sediment; ○, 105 000g supernatant fraction; ●, 150 000g supernatant fraction.

Fig. 3. Incorporation of [^3H]UTP by fractions of ascites-tumour cells obtained by sucrose-density-gradient centrifugation. (a) A microsomal fraction (1.5 ml. containing 20–30 mg. of protein) was centrifuged on a 5–30% (w/v) sucrose gradient at 27000g for 6 hr. in a Spinco S.W.25 rotor. Fractions (1 ml.) were collected and 0.3 ml. portions were assayed under the standard conditions described in Fig. 1. (b) The microsomal fraction was dialysed against 0.01 M-tris-HCl buffer, pH 8.0, at 2–4° for 12 hr. before centrifugation and assay as in (a) above. ●, E at 260 m μ ; ○, E at 280 m μ ; ▲, radioactivity.

Table 1. *Effect of changes in the composition of the reaction mixture on the incorporation of [^3H]ATP, [^3H]GTP, [^3H]CTP and [^3H]UTP by an acetone-dried powder of Landschutz ascites-tumour cells*

The complete system contained: 50 μmoles of tris-HCl buffer, pH 8.5; 5 μmoles each of MgCl_2 and 2-mercapto-ethanol; 0.5 μmole of EDTA; 0.1 μmole of [^3H]ATP, [^3H]GTP, [^3H]CTP or [^3H]UTP; 2.0 μmoles of phosphocreatine; 50 $\mu\text{g.}$ of phosphocreatine kinase; 100 $\mu\text{g.}$ of RNA and 1.5 mg. of acetone-dried powder. Where indicated, 0.25 $\mu\text{g.}$ of ribonuclease, 2.5 $\mu\text{g.}$ of deoxyribonuclease, 20 $\mu\text{g.}$ of actinomycin D, 100 $\mu\text{g.}$ of DNA and 0.1 μmole of each of the other unlabelled ribonucleoside 5'-triphosphates were added. The total volume was 0.5 ml. and incubations were performed at 37° for 20 min.

Reaction conditions	Incorporation of [^3H]ribonucleotide ($\mu\text{moles/mg.}$ of protein)			
	ATP	GTP	CTP	UTP
Complete system	518	97	313	204
+ ribonuclease	81	47	76	49
+ deoxyribonuclease	539	80	300	172
– RNA	379	68	194	91
– RNA + DNA	398	53	154	80
– Mg^{2+}	4	0	0	0
+ actinomycin D	593	83	306	212
+ three unlabelled complementary ribonucleoside 5'-triphosphates	416	43	226	101
– creatine phosphate/creatine phosphokinase	214	51	226	96
– creatine phosphate/creatine phospho- kinase + three unlabelled complementary ribonucleoside 5'-triphosphates	238	29	208	73

of various factors on the incorporation of individual ribonucleotides. The system shows an absolute requirement for Mg^{2+} ions which cannot be replaced by Mn^{2+} ions. The latter are inhibitory in the presence of Mg^{2+} ions even at pH 7.5, where manganese salts are not precipitated. The system is

also stimulated by added RNA and not DNA. The reaction is inhibited by the presence of the other three complementary ribonucleoside 5'-triphosphates, though this effect is not so marked in the absence of a phosphate-regenerating system. The latter considerably stimulates the incorporation of

each labelled ribonucleoside 5'-triphosphate. Paper chromatography of the acid-soluble nucleotides at the end of the reaction showed that there was little interconversion of $\text{ATP} \rightleftharpoons \text{GTP}$ or $\text{CTP} \rightleftharpoons \text{UTP}$ and that such reactions could be disregarded in interpreting the results of incorporating experiments.

Fig. 4 shows the dependence of the incorporation of $[\text{H}]$ ATP, $[\text{H}]$ GTP and $[\text{H}]$ CTP on protein concentration. This is nearly linear up to 1 mg. of protein/reaction tube for each nucleotide under the conditions employed. All subsequent experiments were performed with 0.6–0.7 mg. of protein/tube. Fig. 5 shows the effect of pH on the incorporation of $[\text{H}]$ ATP, $[\text{H}]$ GTP and $[\text{H}]$ CTP. The incorporation of $[\text{H}]$ ATP shows a maximum at pH 8.5, while the incorporations of $[\text{H}]$ GTP and $[\text{H}]$ CTP increase slowly over the range pH 7–9.5 but show no clear maxima. Consequently subsequent studies were carried out at pH 8.5. The phosphate-regenerating system appeared to stimulate the incorporation of each nucleotide to the same extent at pH 8.5 and at pH 9.5, so the pH curves are not distorted by inhibition of phosphocreatine kinase at the higher pH.

Although all four ribonucleoside 5'-triphosphates are incorporated into acid-insoluble material there is no stimulation of the uptake of any one ribonucleotide by the presence of the complementary ribonucleotides. Since the substrate concentration required to observe stimulation of one nucleotide in the presence of three complementary nucleotides

might have been critical, the incorporation of $[\text{H}]$ ATP alone at increasing concentration and in the presence of GTP, CTP and UTP at similar increasing concentrations was investigated over the range 20–400 $\mu\text{moles/ml}$. Inhibition of the utilization of $[\text{H}]$ ATP by GTP, CTP and UTP was observed at all concentrations.

The presence of creatine phosphate and creatine kinase invariably stimulated the incorporation reaction, thus suggesting that polynucleotide phosphorylase is not involved and that ribonucleoside 5'-triphosphates rather than the 5'-diphosphates are the actual substrates. This view is supported by the observation that the reaction is more susceptible to inhibition by inorganic pyrophosphate rather than inorganic orthophosphate. Ribonucleoside 5'-triphosphates rather than deoxyribonucleoside 5'-triphosphates appear to be the preferred substrates for the reaction. Experiments with $[\text{H}]$ deoxyadenosine 5'-triphosphate and thymidine 5'- $[\alpha\text{-}^{32}\text{P}]$ triphosphate showed that these were utilized to less than 5% of the extent of $[\text{H}]$ ATP and $[\text{H}]$ UTP.

Since the incorporation of any one labelled ribonucleotide is not stimulated by the presence of complementary ribonucleotides it seemed unlikely that the reaction represented genuine synthesis of RNA. An alternative possibility is that individual nucleotides are incorporated into homopolyribonucleotides. The results of an experiment showing the distribution of ^{32}P amongst ribonucleoside 3'(2')-monophosphate residues obtained on alkaline

Fig. 4. Effect of enzyme concentration on the incorporation of $[\text{H}]$ ATP, $[\text{H}]$ GTP and $[\text{H}]$ CTP by an acetone-dried powder from the microsomal fraction of Landschutz ascites-tumour cells. The standard reaction mixture and conditions (as in Fig. 1) were employed and enzyme was added as indicated. $\blacktriangle$, $[\text{H}]$ ATP; $\bullet$, $[\text{H}]$ CTP; $\circ$, $[\text{H}]$ GTP.

Fig. 5. Effect of pH on the incorporation of $[\text{H}]$ ATP, $[\text{H}]$ GTP and $[\text{H}]$ CTP by an acetone-dried powder of the microsomal fraction of Landschutz ascites-tumour cells. The standard reaction mixture and conditions (as in Fig. 1) were employed except that the buffer was replaced by 50 μmoles of tris-HCl buffer, of the pH indicated. $\blacktriangle$, $[\text{H}]$ ATP; $\bullet$, $[\text{H}]$ CTP; $\circ$, $[\text{H}]$ GTP.

hydrolysis of the product after the incorporation of [α - 32 P]UTP is shown in Table 2. The 32 P is recovered predominantly in 3'(2')-UMP residues and the distribution is not significantly altered if ATP, GTP and CTP are present in the reaction mixture. Such experiments suggest the formation of poly-uridyate chains.

The nature of the polyribonucleotide product was further investigated by extracting labelled RNA from the reaction mixtures and submitting it to analysis on sucrose-density gradients. The results of such an experiment are shown in Fig. 6 for

Table 2. *Distribution of 32 P amongst the ribonucleoside 3'(2')-monophosphates of the product RNA after the incorporation of [α - 32 P]UTP by an acetone-dried powder of the microsomal fraction of Landschutz ascites-tumour cells*

The standard reaction mixture and conditions (as in Fig. 1) were employed. Where indicated 0.1 μ mole of ATP, GTP and CTP was added. RNA in washed precipitates was hydrolysed with 0.5 ml. of 0.3 N-KOH. Nucleoside monophosphates were separated by paper electrophoresis in 0.05 M-sodium citrate buffer, pH 3.4.

Reaction conditions	Percentage of 32 P in each nucleotide			
	CMP	AMP	GMP	UMP
[α - 32 P]UTP	6.9	12.9	8.8	71.2
[α - 32 P]UTP + ATP, GTP and CTP	7.1	7.2	6.6	79.0

[3 H]UTP, and very similar results were obtained with [3 H]ATP and [3 H]CTP. The 30s and 18s peaks of ribosomal RNA are not labelled whether primer RNA is present or not. All the labelled material is concentrated between the 4s region and the meniscus. Ribonuclease treatment causes a slight shift in the labelled product nearer to the top of the gradient, indicating some breakdown of the reaction product concomitant with disappearance of the 30s and 18s peaks and appearance of ultraviolet-absorbing material at the top of the tube. Such experiments indicate synthesis of separate homopolyribonucleotide chains of relatively small size or labelling of transfer RNA. The latter possibility seems unlikely since only small amounts of transfer RNA are present in the reaction mixtures. Furthermore, a comparison of the priming ability of transfer RNA and ribosomal RNA for [3 H]CTP and [3 H]UTP incorporation showed that transfer RNA does not stimulate the incorporation of [3 H]UTP, although, as might be expected, it stimulates the incorporation of [3 H]-CTP to a greater extent than does ribosomal RNA. This is presumably due to synthesis of the pCpCpA sequence by the terminal addition enzyme.

The average chain lengths of the homopolyribonucleotide sequences synthesized were determined from the ratio of radioactivity present in ribonucleosides and ribonucleotides obtained on alkaline hydrolysis of the reaction products. Table 3 shows the average chain lengths of the products obtained after the incorporation of [3 H]ATP, [3 H]GTP, [3 H]CTP and [3 H]UTP for different

Fig. 6. Analysis on sucrose-density gradients (5–20%, w/v) of [3 H]UMP-labelled RNA extracted from the reaction mixture containing a microsomal fraction of Landschutz ascites-tumour cells. The standard reaction mixture described in Fig. 1 was employed. Microsomal protein (1 mg.) was added and incubations were performed at 37° for 1 hr. RNA was extracted from the reaction mixtures as described in the Experimental section. Centrifugation was at 41000g for 12 hr. (a) Zero time; (b) [3 H]UTP and primer RNA; (c) [3 H]UTP and primer RNA product treated with ribonuclease. In (c), RNA extracted from one tube was treated with 2 μ g. of ribonuclease and incubated at 37° for 45 min. before centrifugation. ●, E at 256 m μ ; ■, radioactivity.

Table 3. Average chain length of homopolyribonucleotides formed by a microsomal fraction from Landschutz ascites-tumour cells

The standard reaction mixture and conditions (as in Fig. 1) were employed. Where indicated 0.1 μ mole of [3 H]-ATP, [3 H]GTP, [3 H]CTP and [3 H]UTP were added. RNA in washed precipitates was hydrolysed with 0.5 ml. of 0.3N-KOH. Ribonucleosides were separated from ribonucleotides by paper chromatography with water-propan-2-ol-aq. NH_3 soln., sp.gr. 0.88 (15:85:1, by vol.) as solvent.

Incubation time (min.) ... Substrate	Chain length		
	15	30	60
[3 H]ATP	2	2	2 or 3
[3 H]GTP	3 or 4	4 or 5	7
[3 H]CTP	2	3	3 or 4
[3 H]UTP	6 or 7	7 or 8	7 or 8

Fig. 7. Nuclease activity of a microsomal fraction of Landschutz ascites-tumour cells with RNA as substrate. The standard reaction mixture (as in Fig. 1) was employed except that ribonucleoside 5'-triphosphates were omitted. Where indicated, 100 μ g. of RNA and 20 μ g. of bentonite were added: ▲, + RNA and bentonite; ■, + RNA; ○, no RNA added. Incubations were performed at 37° for the times indicated.

incubation times. In all cases the chain length is less than eight ribonucleotide residues, polyadenylate being the shortest. These results also suggest that the reaction product is a relatively small polymer and are compatible with the analyses on sucrose-density gradients.

The results of the above experiments are likely to be complicated by the presence of nuclease activity in the microsomal fraction. Measurements of nuclease activity were carried out under the con-

ditions used for the incorporation of ribonucleotides into acid-insoluble material except that ribonucleoside 5'-triphosphates were omitted. The time-course of release of acid-soluble nucleotides from endogenous RNA and from exogenous RNA in the presence and absence of bentonite is shown in Fig. 7. It is clear that the activity of nucleases in the microsomal fraction towards exogenous RNA exceeds the ability of the fraction to promote the incorporation of nucleotides into acid-insoluble material by a factor of at least 100, although hydrolysis of endogenous RNA proceeds much more slowly. Both *p*-nitrophenyl thymidine 3'-phosphate and *p*-nitrophenyl thymidine 5'-phosphate are effective substrates for the nucleolytic enzymes in this fraction, indicating that 3'- and 5'-phosphodiesterases are present (Razzel & Khorana, 1959, 1961).

DISCUSSION

The results described above demonstrate that the microsomal fraction of Landschutz ascites-tumour cells catalyses the independent incorporation into polyribonucleotides of AMP, GMP, CMP and UMP residues from the corresponding ribonucleoside 5'-triphosphates. The reaction is promoted by the addition of RNA and not DNA, is inhibited by ribonuclease but not by deoxyribonuclease or actinomycin D, requires Mg^{2+} rather than Mn^{2+} ions and is inhibited to some extent by the addition of the three complementary ribonucleoside 5'-triphosphates. It can therefore be distinguished sharply from DNA-primed RNA polymerases occurring in bacteria (Krakow & Ochoa, 1963; Nakamoto, Fox & Weiss, 1964; Stevens & Henry, 1964), and in the chromatin fraction from plant and animal sources (Huang & Bonner, 1962; Ro & Busch, 1964; Eason & Smellie, 1965). It has some features in common with the RNA-primed RNA polymerases found on viral infection of bacteria (Haruna, Nozu, Ohtaka & Spiegelman, 1963; Weissman, Borst, Burdon, Billeter & Ochoa, 1964) or animal cells (Baltimore & Franklin, 1963; Baltimore, Eggers, Franklin & Tamm, 1963; Cline, Eason & Smellie, 1963; Eason & Smellie, 1965). In contrast with the last-named enzymes, however, the microsomal system shows no stimulation of the incorporation of any one labelled ribonucleotide by the presence of the three complementary ribonucleotides. Moreover, the distribution of ^{32}P amongst the ribonucleoside 2'(3')-monophosphates obtained on alkaline hydrolysis of the product after the incorporation of [α - ^{32}P]UTP is far from random and becomes no more random when the three complementary ribonucleotides are present in the reaction mixture. Such experiments suggest that the products of the

reactions catalysed by the microsomal fraction are predominantly homopolymers.

Sucrose-density-gradient analysis of RNA extracted from reaction mixtures shows that the endogenous ribosomal RNA does not become labelled and suggests that new polyribonucleotide chains are formed. The synthesis of independent homopolyribonucleotide chains would not account for the appearance of ^{32}P in 3'(2')-CMP, 3'(2')-AMP or 3'(2')-GMP residues obtained on alkaline hydrolysis of RNA after [$\alpha\text{-}^{32}\text{P}$]UTP incorporation (see Table 2). Such labelling might be accounted for by (a) the addition of one or more labelled UMP residues to the 3'-hydroxyl end of some pre-existing RNA, or (b) by the formation in addition to polyuridylyate chains of small amounts of an RNA-like heteropolymer containing all four ribonucleotides. This latter possibility seems unlikely in view of the lack of stimulation by the addition of ATP, GTP and CTP to the reaction mixtures (Table 1) and the observation (Table 2) that, in the presence of the complementary ribonucleoside triphosphates, there is no increase in the relative amounts of radioactivity recovered in the 3'(2')-AMP, 3'(2')-GMP and 3'(2')-CMP residues. Furthermore, the extent of such a reaction would have to be very limited to account for the relatively small amounts of ^{32}P recovered in the 3'(2')AMP, 3'(2')-GMP and 3'(2')-CMP residues.

The first possibility (a) seems more likely to be correct. However, no labelling of endogenous ribosomal RNA or added ribosomal primer is observed (Fig. 6b) and virtually all the labelled product sediments in the 4s region of the sucrose-density gradient. It may be that the RNA which acts as a primer in this reaction is particularly susceptible to degradation by nucleases. If this were so it is surprising that there is relatively little ultraviolet-absorbing material in the region of the sucrose gradient (Fig. 6b) coincident with the incorporated radioactivity since it is clear (Figs. 6a and 6b) that a substantial proportion of the ribosomal RNA has been destroyed during the incubation period. It may be that small oligonucleotides formed by the action of nucleases on endogenous and exogenous RNA serve as initiators for the synthesis of new homopolyribonucleotides. Klemperer (1964) has shown that short oligonucleotides are the most effective primers for the synthesis of polyadenylate by a partially purified enzyme from rat-liver cytoplasm.

The observations of short average chain length and small size of the products of the reaction described above (Table 3; Fig. 6) may be accounted for by virtue of nucleolytic degradation of the reaction product by endonucleases and exonucleases present in the microsomal fraction.

Other mammalian enzymes synthesizing homo-

polyribonucleotides in RNA-primed reactions occur in pigeon-liver microsomes (Straus & Goldwasser, 1961), in a cytoplasmic fraction from rat liver (Klemperer, 1963a,b), in calf-thymus nuclei (Edmonds & Abrams, 1962; Abrams, Edmonds & Biswas, 1962) and in the chlorioallantoic membranes of chick embryos (Venkataraman & Mahler, 1963). Some of these enzymes have been partially purified and are specific for the incorporation of single ribonucleoside 5'-triphosphates into existing primer RNA chains. In these systems there is variable inhibition of the incorporation of specific ribonucleoside 5'-triphosphates by the presence of the complementary ribonucleoside 5'-triphosphates, but Mg^{2+} ions are usually required. There is little specificity for the primer RNA though the extent of incorporation often varies considerably with synthetic polyribonucleotides and RNA from different sources. This is in contrast with the specific requirement for transfer RNA by an enzyme purified from the cell sap and catalysing the formation of the pCpCpA terminal sequence of these molecules (Hecht, Zamecnik, Stephenson & Scott, 1958; Preiss, Dieckmann & Berg, 1961).

The microsomal enzyme differs from enzymes of the above type in that four ribonucleoside 5'-triphosphates are independently incorporated into homopolyribonucleotides and the RNA requirement differentiates it from enzymes requiring DNA as a primer for the synthesis of homopolyribonucleotides (Chambon, Weill & Mandel, 1963; Burdon, 1963).

No evidence has been obtained for the occurrence in the cytoplasm of Landschutz ascites-tumour cells of an enzyme catalysing the synthesis of RNA by replication of an RNA template. This is in agreement with the results of several types of experiments which suggest that messenger RNA, ribosomal RNA and transfer RNA are all synthesized in the nucleus on a DNA template. However, the results of the present experiments do show that cytoplasmic fractions are capable of limited synthesis of homopolyribonucleotides *in vitro*. It is difficult to ascribe any function to these systems at the present time.

We express our thanks to Professor J. N. Davidson, F.R.S., for making the necessary facilities available to us for this work with the aid of grants from the British Empire Cancer Campaign and the Rankine Fund of the University of Glasgow. We also thank the Medical Research Council for a grant to R. M. S. S. and for a Scholarship to J. R. W.

REFERENCES

- Abrams, R., Edmonds, M. & Biswas, B. B. (1962). *Colloq. int. Cent. nat. Rech. sci.*, no. 106: *Acides Ribonucléiques et Polyphosphates; Structure, Synthèse et Fonction*, Strasbourg, p. 323. Paris: Centre National de la Recherche Scientifique.

- Baltimore, D., Eggers, H. J., Franklin, R. M. & Tamm, I. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 843.
- Baltimore, D. & Franklin, R. M. (1963). *J. biol. Chem.* **238**, 3395.
- Britten, R. J. & Roberts, R. B. (1960). *Science*, **131**, 32.
- Burdon, R. H. (1963). *Biochem. biophys. Res. Commun.* **13**, 37.
- Burdon, R. H. & Smellie, R. M. S. (1961). *Biochim. biophys. Acta*, **51**, 153.
- Burdon, R. H. & Smellie, R. M. S. (1962). *Biochim. biophys. Acta*, **61**, 633.
- Chambon, P., Weill, J. D. & Mandel, P. (1963). *Biochem. biophys. Res. Commun.* **11**, 39.
- Cline, M. J., Eason, R. & Smellie, R. M. S. (1963). *J. biol. Chem.* **238**, 1788.
- Eason, R., Cline, M. J. & Smellie, R. M. S. (1963). *J. biol. Chem.* **238**, 3978.
- Eason, R. & Smellie, R. M. S. (1965). *J. biol. Chem.* **240**, 2580.
- Edmonds, M. & Abrams, R. (1962). *J. biol. Chem.* **237**, 2636.
- Fisher, H. W. & Harris, H. (1962). *Proc. Roy. Soc. B*, **156**, 521.
- Goodman, H. M. & Rich, A. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2101.
- Haruna, I., Nozu, K., Ohtaka, Y. & Spiegelman, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **50**, 905.
- Hecht, L. I., Zamecnik, P. C., Stephenson, M. L. & Scott, J. F. (1958). *J. biol. Chem.* **233**, 954.
- Huang, R. C. & Bonner, J. (1962). *Fed. Proc.* **21**, 384.
- Klempere, H. G. (1963a). *Biochim. biophys. Acta*, **72**, 403.
- Klempere, H. G. (1963b). *Biochim. biophys. Acta*, **72**, 416.
- Klempere, H. G. (1964). *Biochem. biophys. Res. Commun.* **15**, 269.
- Krakow, J. S. & Ochoa, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 88.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Nakamoto, T., Fox, C. F. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 167.
- Perry, R. P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 2179.
- Perry, R. P., Hell, A. & Errera, M. (1961). *Biochim. biophys. Acta*, **49**, 47.
- Preiss, J., Dieckmann, M. & Berg, P. (1961). *J. biol. Chem.* **236**, 1748.
- Razzel, W. E. & Khorana, H. G. (1959). *J. biol. Chem.* **234**, 2105.
- Razzel, W. E. & Khorana, H. G. (1961). *J. biol. Chem.* **236**, 1144.
- Ro, T. S. & Busch, H. (1964). *Cancer Res.* **24**, 1630.
- Scherrer, K., Latham, H. & Darnell, J. E. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 240.
- Smith, M. & Khorana, H. G. (1958). *J. Amer. chem. Soc.* **80**, 1141.
- Stevens, A. & Henry, J. (1964). *J. biol. Chem.* **239**, 196.
- Straus, D. B. & Goldwasser, E. (1961). *J. biol. Chem.* **236**, 849.
- Tener, G. M. (1961). *J. Amer. chem. Soc.* **83**, 159.
- Venkataraman, P. R. & Mahler, H. R. (1963). *J. biol. Chem.* **238**, 1058.
- Weiss, S. B. (1960). *Proc. nat. Acad. Sci., Wash.*, **46**, 1020.
- Weissmann, C., Borst, P., Burdon, R. H., Billeter, M. A. & Ochoa, S. (1964). *Proc. nat. Acad. Sci., Wash.*, **51**, 890.
- Yanofsky, S. A. & Spiegelman, S. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 1466.
- Yanofsky, S. A. & Spiegelman, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 538.