The Incorporation of Cytidine 5'-Monophosphate and other Ribonucleotides by an Enzyme Preparation from Rat-Liver Microsomes

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(Received 14 June 1966)

1. An enzyme preparation from rat-liver microsomes incorporated all four ribonucleotides from the corresponding triphosphates into ribosomal RNA. The reaction was Mn^{2+} -dependent, but UMP incorporation also occurred in the presence of Mg^{2+} . 2. The incorporation of any one ribonucleotide was inhibited by the presence of the other three ribonucleoside triphosphates and by denatured DNA. 3. The product of the reaction consisted of short chains of homopolymer attached to the primer ribosomal RNA. 4. 'Soluble' RNA, synthetic polyribonucleotides, and oligoribonucleotides were also effective primers for CMP incorporation. 5. When phosphodiesterase-treated 'soluble' RNA was the primer, CMP was incorporated into positions usually occupied by the normal terminal trinucleotide sequence of intact 'soluble' RNA, but the enzyme did not synthesize a specific terminal sequence consisting of a defined number of CMP residues.

In the presence of Mg^{2+} microsomes from pigeon liver and from tumour cells incorporate UMP and AMP from the corresponding ribonucleoside triphosphates into chains of polyU* and polyA attached to the 3'-ends of RNA (Straus & Goldwasser, 1961; Burdon, 1963a; Dalgarno, Martin, Liu & Work, 1966). The incorporation of all four ribonucleotides in a Mg^{2+} -dependent reaction also occurs in microsomes from tissue-culture cells (Baltimore & Franklin, 1963) and from tumour cells (Smellie, 1965), and Wykes & Smellie (1966) have shown that the products of the reaction are short homopolymer chains attached to RNA.

Enzymes that attach UMP as chains of polyU to the 3'-ends of primer RNA have been isolated from the soluble fraction of animal cells (Canellakis, 1957; Burdon & Smellie, 1961; Klemperer, 1963a). Similar enzymes for the attachment of AMP exist in the soluble fraction of animal cells (Chung, Mahler & Enrione, 1960; Venkataraman & Mahler, 1963; Klemperer, 1963b), in bacteria (August, Ortiz & Hurwitz, 1962; Gottesman, Canellakis & Canellakis, 1962) and in thymus nuclei (Edmonds & Abrams, 1960, 1962). Recently the corresponding enzymes have been isolated from thymus nuclei

for CMP incorporation (Edmonds, 1965) and from chloroplasts for GMP incorporation (Chakravorty & Biswas, 1965).

The present paper reports a Mn^{2+} -dependent incorporation of all four ribonucleotides into RNA by rat-liver microsomes. In order to define the reaction further, particularly with respect to CMP incorporation, the enzyme was prepared in a form that was free of nucleases and of RNA, but this resulted in very large losses of total activity with only a small increase in specific activity.

MATERIALS AND METHODS

Materials. ATP, CTP, GTP and UTP were purchased from Calbiochem Ltd. (Basingstoke, Hants.). Solutions of the required molarity were adjusted to pH 7.0 with 1.0N-NaOH. Creatine phosphate was purchased from Boehringer Corporation Ltd. (Ealing, London, W. 5). Hydroxyapatite suspension was obtained from Calbiochem Ltd.

[8- ^{14}C]ATP and [8- ^{14}C]GTP were purchased from Schwartz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). [6- ^{14}C]CTP and [6- ^{14}C]UTP were prepared by the enzymic method of Canellakis, Gottesman & Kammen (1960) from [6- ^{14}C]orotic acid purchased from The Radiochemical Centre (Amersham, Bucks.). [6- ^{14}C]CDP was a by-product isolated during the preparation of the [6- ^{14}C]CTP. The specific activities (counts/min./ μ mole) of these materials determined by liquid-scintillation counting (see below) were: ATP, 6.6×10^6 ; CTP, 6.0×10^6 ; GTP, 7.8×10^6 ; UTP, 6.7×10^6 ; CDP, 3.1×10^6 .

Enzymes. Creatine phosphokinase (EC 2.7.3.2) was prepared by the method of Kuby, Noda & Lardy (1954).

* Abbreviations: polyA, polyadenylic acid; polyC, polycytidylic acid; polyI, polyinosinic acid; polyU, polyuridylic acid; ApApA, triadenosine diphosphate; s-RNA, 'soluble' RNA; s-RNA-pCpCpA, s-RNA bearing the terminal-CMP-CMP-AMP sequence; s-RNA-pC, the same but bearing only the innermost residue of this sequence.

Crystalline pancreatic deoxyribonuclease (EC 3.1.4.5) was purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Snake-venom phosphodiesterase (EC 3.1.4.1) was purified from *Crotalus adamanteus* venom (Sigma Chemical Co., London, S.W. 6), by the method of Koerner & Sinsheimer (1957) and had an activity of 4000 units/ml. s-RNA nucleotidyltransferase (EC 2.7.7.25) was prepared from rat liver as described by Canellakis & Herbert (1960).

Nucleic acids. Calf-thymus DNA from Worthington Biochemical Corp. was dissolved at 1 mg./ml. in 0.02 M-tris-HCl buffer, pH 7.4. Denatured DNA was prepared by heating this solution for 30 sec. at 100° and then cooling rapidly in ice.

The synthetic polyribonucleotides polyA, polyC, polyI and polyU were obtained from Miles Laboratories (Clifton, N.J., U.S.A.). Immediately before use the solution of polyI (200 µg./ml. in 0.02 M-tris-HCl buffer, pH 7.4) was heated for 30 sec. at 100° and then cooled rapidly in ice. The oligoribonucleotide ApApA was prepared from polyA as described by Klemperer (1965).

Ribosomal RNA was made by phenol extraction of ribonucleoprotein particles prepared by deoxycholate treatment of rat-liver microsomes (e.g. Klemperer, 1963a). The solution in 0.02 M-tris-HCl buffer, pH 7.4, was freed from any low-molecular-weight polyribonucleotide by passage over a column (1.5 cm. × 25 cm.) of Sephadex G-200 (Pharmacia Ltd., London, W. 13).

s-RNA was made by phenol extraction of the pH 5 precipitate prepared from the 105000g supernatant of rat-liver homogenate (e.g. Klemperer, 1963a). The solution (15 ml.) containing 1.0 mg. of RNA/ml. in 0.02 M-tris-HCl buffer, pH 7.4, was applied to a column (1.5 cm. × 3 cm.) of DEAE-cellulose powder (Whatman DE50; Reeve Angel and Co., London, E.C. 4) that had previously been washed with 0.02 M-tris-HCl buffer, pH 7.4. The column was washed with 12 ml. of 0.3 M-NaCl-0.01 M-tris-HCl buffer, pH 7.4, and the s-RNA was then eluted in 8 ml. of 1.0 M-NaCl (Rosset, Monier & Julien, 1964) and dialysed against 0.02 M-tris-HCl buffer, pH 7.4. The product had a total capacity for 0.5 µmole of CMP and 4.0 µmoles of AMP/mg. when incubated with s-RNA nucleotidyltransferase as described by Herbert & Canellakis (1961).

Phosphodiesterase-treated s-RNA was prepared by incubating s-RNA with snake-venom phosphodiesterase as described by Herbert & Canellakis (1961). The product had a total capacity of 19 µmoles of CMP and 9.5 µmoles of AMP/mg. when incubated with s-RNA nucleotidyltransferase.

To prepare s-RNA-pCpCpA labelled with [¹⁴C]AMP in the terminal residue, 60 µg. of phosphodiesterase-treated s-RNA was incubated in a final volume of 1 ml. with s-RNA nucleotidyltransferase (400 µg. of protein), 5 µmoles of MgCl₂, 150 µmoles of CTP, 150 µmoles of [¹⁴C]ATP, 100 µmoles of tris-HCl buffer, pH 8.0, and 5 µmoles of mercaptoethanol for 60 min. at 37°. Under these conditions the s-RNA became saturated with -pCpCpA terminal groups. At the end of the incubation the labelled s-RNA-pCpCpA was separated from [¹⁴C]ATP by passing the whole reaction mixture over a column (20 cm. × 1 cm.) of Sephadex G-25. The RNA together with protein was eluted in the first 1 ml. after the dead space. This eluate was heated for 90 sec. at 100° to inactivate the enzyme and used directly as a source of labelled s-RNA-pCpCpA.

Where s-RNA-pCpC labelled with [¹⁴C]CMP was prepared, the reaction mixture was the same as above except that [¹⁴C]CTP was used and ATP was omitted.

Incubation for incorporation of [¹⁴C]ribonucleotide into RNA. The reaction mixture contained (in 1 ml.) 30 µmoles of [¹⁴C]ribonucleoside triphosphate, 50 µg. of ribosomal RNA, 2 µmoles of MnCl₂, 10 µmoles of mercaptoethanol, 100 µmoles of tris-HCl buffer, pH 8.0, and purified microsomal enzyme (50–100 µg. of protein). After incubation for 30 min. at 37° the tubes were cooled in ice and 0.3 ml. of 0.2 M-sodium pyrophosphate buffer, pH 7.5, was added to discharge non-enzymically bound ribonucleotides (Kammen, Klemperer & Canellakis, 1961). The acid-insoluble radioactivity was precipitated with 0.6 ml. of 50% (w/v) trichloroacetic acid in the presence of 100 µg. of ribosomal RNA and 200 µg. of bovine serum albumin. After standing at 0° for 30 min. the precipitate was collected by filtration (Josse & Kornberg, 1962) on 2.5 cm.-diam. glass-paper disks (Whatman GF/C) and washed on the filter three times with 5 ml. quantities of 6% (w/v) trichloroacetic acid at 0°. The disks were dried for 16 hr. in an oven at 37° and then placed in vials together with 10 ml. of scintillation fluid [toluene containing 4 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)-benzene/l.] for measurement of radioactivity in a liquid-scintillation counter (background 35 counts/min.; efficiency approx. 75%).

When ApApA was the primer the reaction was stopped by heating for 90 sec. at 100°, and chromatography on DEAE-cellulose paper (Whatman DE81) was used to separate the reaction product (Klemperer, 1965). Radioactivity in the paper area containing the reaction product was measured in the same way as on the glass-paper disks.

Incubation with s-RNA nucleotidyltransferase. The reaction mixture contained (in 1 ml.) 5 µg. of s-RNA, 60 µmoles of [¹⁴C]CTP, 10 µmoles of MgCl₂, 5 µmoles of mercaptoethanol, 100 µmoles of tris-HCl buffer, pH 8.0, and s-RNA nucleotidyltransferase (50 µg. of protein). When this incubation was preceded by an incubation with the microsomal enzyme, the latter was present in a final volume of 0.5 ml. containing 5 µg. of s-RNA, 30 µmoles of [¹⁴C]CTP, 1 µmole of MnCl₂, 5 µmoles of mercaptoethanol and 50 µmoles of tris-HCl buffer, pH 8.0. This preliminary incubation was terminated by heating for 90 sec. at 100°. The components for the s-RNA-nucleotidyltransferase reaction were then added as above and the volume of the mixture was adjusted to 1 ml.

Location of [¹⁴C]ribonucleotide in reaction product. The acid-insoluble reaction product was collected by centrifugation (10 min. at 2000g) and was washed three times by resuspension in 8 ml. of 0.4 N-HClO₄ followed by centrifugation. The precipitate was hydrolysed in 0.3 N-KOH for 16 hr. at 37°. The ribonucleosides and 2'(3')-monoribonucleotides that were formed under these conditions were separated in the presence of added non-radioactive carrier compounds by stepwise elution from columns (1 cm. × 3 cm.) of Dowex 1 (formate form) as described by Hecht, Zamecnik, Stephenson & Scott (1958). Samples (2 ml.) from the eluates were dried in stainless-steel planchets at 80° and the radioactivity was measured at zero thickness in a windowless gas-flow counter (background 16 counts/min.; efficiency approx. 30%).

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

with bovine serum albumin as the standard, and from the extinctions at 280 and 280 $m\mu$ (Layne, 1957).

Purification of microsomal enzyme. Rat liver (approx. 60 g. from six animals) was dispersed in 2.5 vol. of 0.35 M-sucrose-0.02 M-tris-HCl buffer, pH 8.0, with 12 strokes at 2500 rev./min. in a glass homogenizer fitted with a Teflon pestle (0.25 mm. clearance). The homogenate was centrifuged twice for 10 min. at 10000g and the supernatant from the second centrifugation was then centrifuged for 90 min. at 105000g to sediment the microsome fraction.

(I) Extraction of microsomes. This was based on the procedure applied by August *et al.* (1962) to *Escherichia coli* ribosomes. The microsomes were suspended with a homogenizer in 150 ml. of 0.005 M-glycylglycine-0.05 M-tris-HCl buffer, pH 8.7. The mixture was stirred for 20 min. at 0° and then centrifuged for 60 min. at 105000g. The supernatant was withdrawn, adjusted to 0.005 M-mercaptoethanol and brought to pH 7.6 (glass electrode) by the dropwise addition of 0.5 N-acetic acid.

(II) Ammonium sulphate precipitation and acetone treatment. The microsome extract was stirred at 0° and $(\text{NH}_4)_2\text{SO}_4$ (351 g./l.) was added. After an equilibration period of 10 min. the precipitate was collected by centrifugation for 10 min. at 10000g, dispersed in 200 ml. of acetone at -20° and then collected by filtration. The powder was washed on the filter with a further 200 ml. of acetone, dried by drawing air at 4° through the filter and then dissolved in 40 ml. of 0.01 M-mercaptoethanol-0.05 M-tris-HCl buffer, pH 7.4. The solution was dialysed overnight against 0.005 M-mercaptoethanol-0.02 M-tris-HCl buffer, pH 7.4. Traces of insoluble material were then removed by centrifugation. Acetone treatment of the $(\text{NH}_4)_2\text{SO}_4$ precipitate increased the yield of enzyme in the next step.

(III) Calcium phosphate-gel adsorption. The solution (40 ml. containing 7.5 mg. of protein/ml.) was stirred at 0° and 1 ml. of 0.2 M-mercaptoethanol, 6 ml. of 0.5 M-sodium phosphate buffer, pH 7.4, and 14 ml. of $\text{Ca}_3(\text{PO}_4)_2$ gel (2.5 g./100 ml.) were added in succession. The mixture was stirred for 10 min. and then centrifuged for 10 min. at 10000g. The precipitate was resuspended in 40 ml. of 0.01 M-mercaptoethanol-0.05 M-sodium phosphate buffer, pH 7.4, and spun down as before. The supernatant was discarded and the precipitate was suspended in 25 ml. of 0.01 M-mercaptoethanol-0.05 M-tris-HCl buffer, pH 7.4, containing 10% (w/v) of $(\text{NH}_4)_2\text{SO}_4$. The suspension was stirred for 5 min. at 0° and the eluate, which contained the enzyme, was collected after centrifugation.

(IV) Second ammonium sulphate precipitation. $(\text{NH}_4)_2\text{SO}_4$ (213 g./l.) was added to the eluate with stirring. After equilibration for 10 min. the mixture was centrifuged for 10 min. at 10000g. The precipitate was washed by resuspension in 30 ml. of 0.01 M-mercaptoethanol-0.05 M-tris-HCl buffer, pH 7.4, containing 313 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. (50% saturation) and again spun down. The precipitate was then extracted with 6 ml. of the same medium containing 176 g. of $(\text{NH}_4)_2\text{SO}_4$ /l. (30% saturation). The precipitate was removed by centrifugation and the supernatant, which contained the enzyme, was collected. The $(\text{NH}_4)_2\text{SO}_4$ was then removed by passing the extract over a column (1.5 cm. $\times$ 20 cm.) of Sephadex G-25 that had been equilibrated with 0.01 M-mercaptoethanol-0.02 M-tris-HCl buffer, pH 7.4.

(V) Hydroxyapatite adsorption. The eluate from the

Sephadex G-25 column (8.5 ml. containing a total of 15 mg. of protein) was mixed with 1.5 ml. of 1.0 M-potassium phosphate buffer, pH 6.8. Packed hydroxyapatite (1.5 ml. packed volume; 0.68 g. dry wt.) was then resuspended in the above enzyme solution. The mixture was centrifuged for 10 min. at 2000g, the sediment was resuspended in 10 ml. of 0.01 M-mercaptoethanol-0.15 M-potassium phosphate buffer, pH 6.8, and the mixture was again centrifuged for 10 min. at 2000g. The enzyme was eluted by suspending the precipitate in 6 ml. of 0.01 M-mercaptoethanol-0.05 M-tris-HCl buffer, pH 7.4, containing 10% (w/v) of $(\text{NH}_4)_2\text{SO}_4$. The eluate obtained after removal of the precipitate by centrifugation was passed over Sephadex G-25 to remove $(\text{NH}_4)_2\text{SO}_4$ as in stage (IV).

The resulting enzyme solution (8 ml. containing 0.4 mg. of protein/ml.) when stored at 0° lost 30–50% of its activity for CMP incorporation in 1 week. The enzyme preparation appeared to be free of RNA (E_{280}/E_{260} ratio 1.58) and was completely dependent on the addition of RNA for ribonucleotide incorporation. Neither ribonuclease (McDonald, 1955) nor phosphodiesterase (Koerner & Sinsheimer, 1957) was detectable by the normal methods of determination. Phosphodiesterase activity was, however, detectable by the release of ^{14}C -labelled terminal ribonucleotides from s-RNA (made by incubating phosphodiesterase-treated s-RNA with s-RNA nucleotidyltransferase and ^{14}C CTP or CTP plus ^{14}C ATP). Thus under the conditions shown in Table 6 (80 μg . of enzyme protein and 5 μg . of s-RNA/0.5 ml.) about 30–50% of the ^{14}C CMP was released from s-RNA-pCpC and about 10–20% of the ^{14}C AMP from s-RNA-pCpCpA. There was also about 20% breakdown of CTP to CDP and CMP during incubation for 30 min. with the enzyme under standard conditions (30 μM moles of CTP and 80 μg . of enzyme protein/ml.).

General remarks on enzyme purification. The procedure gave only a five- to ten-fold purification of the activity for CMP incorporation and a very low overall yield (Table 1: 1% of microsomes, 5% of pH 8.7 extract). This is attributed in part to the fact that before stage (III) there is a variable proportion of the enzyme was in a 'soluble' form. After this stage the enzyme activity did not sediment appreciably when layered over a linear 5–20% (w/v) sucrose density gradient and centrifuged for 4 hr. at 37000 rev./min. in an MSE preparative ultracentrifuge, but the enzyme was now very unstable, losing up to 50% of the activity/week at 0°.

RESULTS

Incorporation of ribonucleotides by microsomes. Microsomes incorporated radioactivity into RNA when incubated with any one of the ^{14}C ribonucleoside triphosphates in the presence of Mn^{2+} (Table 1). In other experiments it was shown that this incorporation was unaffected by deoxyribonuclease and was inhibited when the other three non-radioactive ribonucleoside triphosphates were added. A similar enzymic activity was present in an extract prepared from the microsomes at pH 8.7, and procedures that removed nucleases and RNA gave a five- to ten-fold increase in specific activity (Table 1). The relative activities towards the individual ribonucleotides varied somewhat with

Table 1. *Purification of microsomal enzyme*

The enzyme preparation (80–200 μ g. of protein) was incubated for 10 min. at 37° with 30 μ moles of [14 C]-ribonucleoside triphosphate, 50 μ g. of ribosomal RNA, 2 μ moles of $MnCl_2$, 10 μ moles of mercaptoethanol and 100 μ moles of tris-HCl buffer, pH 8.0, in a final volume of 1.0 ml. In addition, 1.5 μ moles of creatine phosphate and 50 μ g. of creatine phosphokinase were added to incubations with microsomes or enzyme at stages (I) and (II). The acid-precipitable radioactivity was collected, washed and counted on glass-paper disks. Total activity (μ moles of CMP/10 min.) refers to material from 60 g. of liver.

Purification stage	Preparation	[14 C]Ribonucleotide incorporated (μ moles/10 min./mg. of protein)				Total activity for CMP
		CMP	AMP	GMP	UMP	
—	Microsomes	50	320	10	15	90.0
(I)	pH 8.7 extract	75	120	20	36	20.0
(II)	Acetone-dried powder of first (NH_4) $_2$ SO $_4$ fraction	40	—	—	—	7.3
(III)	Ca $_3$ (PO $_4$) $_2$ -gel eluate	65	150	35	40	4.8
(IV)	Second (NH_4) $_2$ SO $_4$ fraction	290	—	—	—	2.1
(V)	Hydroxyapatite eluate	410	910	75	320	1.0

Table 2. *Incorporation of ribonucleotides into RNA by microsomal enzyme*

Purified (stage V) microsomal enzyme (80 μ g./ml.) was incubated with labelled ribonucleoside triphosphates under the conditions described in Table 1 for 30 min. at 37°. Where indicated, the other three non-radioactive ribonucleotides (each 30 μ moles/ml.) were added, or $MgCl_2$ (10 μ moles/ml.) replaced $MnCl_2$.

[14 C]Ribonucleoside triphosphate precursor	[14 C]Ribonucleotide incorporated (μ moles/30 min.)		
	Mn $^{2+}$	Mn $^{2+}$ + tri-phosphates	Mg $^{2+}$
ATP	63	14	0
CTP	45	15	1
GTP	12	2	1
UTP	41	35	36

different preparations, but attempts to isolate an enzyme specific for one ribonucleotide only were unsuccessful. The activity of the final preparation (approx. 0.4 μ mole of CMP incorporated/mg./10 min.) may be compared with that of Edmonds' (1965) enzyme preparation, which is specific for CMP and incorporates 0.5 μ mole/mg./10 min. into ribosomal RNA and 3.6 μ moles/mg./10 min. into a primer that is specific to this enzyme.

Incorporation of ribonucleotides by the microsomal enzyme preparation. The partially purified enzyme, like the microsome suspension, incorporated less [14 C]ribonucleotide from [14 C]ribonucleoside triphosphate when the other three non-radioactive ribonucleoside triphosphates were added (Table 2). In the presence of Mg^{2+} only UTP was incorporated.

Particular attention was paid to the incorporation of CMP, which is illustrated by the results in

Table 3. *Incorporation of CMP into RNA*

The reaction mixture contained (in 1 ml.) 30 μ moles of [6- 14 C]CTP, 2 μ moles of $MnCl_2$, 10 μ moles of mercaptoethanol, 100 μ moles of tris-HCl buffer, pH 8.0, 50 μ g. of ribosomal RNA and purified (stage V) enzyme (70 μ g. of protein). These conditions were modified as indicated. When added, DNA was 50 μ g., [14 C]CDP and non-radioactive triphosphates were each 30 μ moles and deoxyribonuclease was 5 μ g. The incorporation of radioactivity into acid-insoluble material was determined after incubating for 30 min. at 37°.

Reaction mixture	Additions	[14 C]CMP incorporated (μ moles)
Complete	—	47
RNA omitted	—	0
RNA omitted	DNA	0
[14 C]CTP omitted	[14 C]CDP	1
Complete	Deoxyribonuclease	50
Complete	DNA	48
Complete	Denatured DNA	25
Complete	ATP, GTP, UTP	17
Complete	ATP, GTP, UTP, DNA	11

Table 3. The optimum conditions were pH 8.0 and the presence of 2 mM-manganese chloride, and the maximum rate of incorporation was attained with about 100 μ M-CTP and 50 μ g. of ribosomal RNA/ml. Under these conditions the reaction rate remained constant for about 45 min. and was proportional to the enzyme concentration (up to 150 μ g. of protein/ml.). Half-maximum velocity was given by about 25 μ M-CTP.

Table 3 shows that the reaction was completely dependent on the addition of RNA. DNA could not replace RNA, and negligible radioactivity was incorporated from [14 C]CDP. Added deoxyribo-

Table 4. *Location of ribonucleotides incorporated into ribosomal RNA*

The reaction mixtures were the same as described in Table 3 except that [^{14}C]CTP was 60 $\mu\text{moles/ml.}$ and was replaced by labelled ATP, UTP or GTP (all 60 $\mu\text{moles/ml.}$) as indicated. RNA was added in the amounts shown. Several replicate tubes were pooled to obtain sufficient radioactivity. In the 70 min. incubation with [^{14}C]CTP an additional 30 μmoles of [^{14}C]CTP/ml. were added after 30 min. incubation. The reaction product was precipitated and washed with 0.4 N-HClO_4 and then hydrolysed for 16 hr. at 37° with 0.3 N-KOH . The digest was fractionated on a Dowex 1 (formate form) column after the addition of carrier compounds. The recovery of radioactivity was essentially complete in all cases.

[^{14}C]Ribonucleoside triphosphate precursor	RNA ($\mu\text{g./ml.}$)	Incubation time (min.)	Radioactivity recovered (%)			Total radioactivity (counts/min.)	Chain length
			In ribo-nucleoside	In ribo-nucleotide	In ribo-nucleoside diphosphate		
CTP	50	30	89	11	<1	960	1.1
CTP	10	70	33	67	<1	702	3.0
ATP	20	60	15	85	<1	835	6.7
UTP	20	60	37	63	<1	783	2.7
GTP	20	60	43	57	<1	400	2.3

nuclease or native DNA had no significant effect, but denatured DNA inhibited the reaction. The addition of ATP, GTP and UTP inhibited [^{14}C]CMP incorporation both in the presence and in the absence of native DNA. In other experiments, not shown here, the incorporation of the other ribonucleotides was found to have essentially similar characteristics.

Location of ribonucleotides incorporated into ribosomal RNA. Table 4 shows that the ribonucleotides were attached to the 3'-hydroxyl groups of the primer RNA. Thus alkaline hydrolysis of the reaction product formed from a single [^{14}C]ribonucleoside triphosphate yielded only the corresponding [^{14}C]ribonucleoside and 2'(3')-[^{14}C]ribonucleotide. These are derived respectively from the 3'-terminal ribonucleotide residue and from internal residues. Since no 2'(3'),5'-[^{14}C]ribonucleoside diphosphate was recovered, the formation of a polynucleotide terminated at the 5'-end by a labelled residue is excluded.

Under the optimum conditions for [^{14}C]CMP incorporation most of the radioactivity was recovered as [^{14}C]cytidine, indicating that mainly single CMP residues were attached to the primer (Table 4). With longer incubations and less RNA, short chains with an average of three CMP residues were synthesized. The fact that only short chains were formed may reflect the presence of diesterase in the enzyme preparation rather than a property of the CMP-incorporating enzyme itself. Similar considerations apply to the products formed from ATP, UTP and GTP, which are also presented in Table 4. In other experiments essentially similar results were also found for UMP incorporated in the presence of Mg^{2+} .

Incorporation of CMP into other polyribonucleotides. The synthetic polyribonucleotides, with the

Table 5. *Incorporation of CMP and AMP into polyribonucleotides*

Tubes were incubated for 30 min. under the conditions described in Table 3 except that ribosomal RNA was 20 $\mu\text{g./ml.}$ and was replaced by other polyribonucleotides (all 20 $\mu\text{g./ml.}$) as indicated. When ApApA (10 $\mu\text{moles/ml.}$) was the primer the reaction was stopped by heating for 90 sec. at 100° and the product was separated by chromatography on DEAE-cellulose paper (Klemperer, 1965).

Polyribonucleotide primer	Ribonucleotide incorporated	
	[^{14}C]CMP (μmoles)	[^{14}C]AMP (μmoles)
Ribosomal RNA	51	125
PolyA	26	32
PolyC	32	—
PolyI	43	—
PolyU	7	15
PolyA + polyU*	17	150
PolyC + polyI*	17	—
ApApA	30	1400
s-RNA (native)	69	—
s-RNA (phosphodiesterase-treated)	70	—

* These are the polyribonucleotides actually added. It is highly probable that in each case the two homopolymers are completely associated by hydrogen-bonding to give poly(A)-(U) and poly(C)-(I) respectively, but there is no direct proof of this in the present instance.

exception of polyU, were fairly effective primers for the incorporation of CMP (Table 5). Double-stranded molecules formed by mixing roughly equivalent amounts of polyA with polyU, or of polyC with polyI, were less effective primers. The oligoribonucleotide ApApA primed the reaction to

Table 6. *Incorporation of CMP into s-RNA*

In the normal procedure s-RNA (5 μ g.) was incubated first with microsomal enzyme for 60 min. at 37°. This reaction was terminated by heating for 90 sec. at 100° (which does not significantly affect the reaction product), and a second incubation followed with s-RNA nucleotidyltransferase for 45 min. at 37°. The reaction mixture for the first incubation contained (in 0.5 ml.) 5 μ g. of s-RNA, 80 μ g. of ribosomal enzyme, 30 μ moles of [14 C]CTP, 1 μ mole of MnCl₂, 5 μ moles of mercaptoethanol and 50 μ moles of tris-HCl buffer, pH 8.0. For the second incubation 50 μ g. of s-RNA nucleotidyltransferase, 30 μ moles of [14 C]CTP, 10 μ moles of MgCl₂, 5 μ moles of mercaptoethanol and 50 μ moles of tris-HCl buffer, pH 8.0, were added and the total volume was increased to 1 ml. These conditions were modified as shown. The incorporation of radioactivity into acid-insoluble material was determined as described in Table 1.

First incubation (microsomal enzyme)	Second incubation (s-RNA nucleotidyltransferase)	[14 C]CMP incorporated (μ moles)
Enzyme omitted	Normal	95
Normal	Not incubated	59
		(Sum = 154)
Normal	Normal	109
		(Deficit = 45)
CTP omitted	Normal	96

about the same extent as polyA. This may be contrasted with the results for the incorporation of [14 C]AMP (also shown in Table 5). In this case polyA plus polyU was a better primer than each polyribonucleotide separately, and ApApA was about ten times as effective as ribosomal RNA. This is very similar to the characteristics previously found for AMP incorporation by an enzyme from rat-liver supernatant (Klempner, 1965).

Table 5 also shows that both native s-RNA and s-RNA that had been treated with phosphodiesterase to remove the terminal -pCpCpA sequence were good primers for CMP incorporation. This reaction was Mn²⁺-dependent: no incorporation occurred when magnesium chloride replaced manganese chloride. In other experiments there was no evidence that s-RNA could be saturated with CMP by prolonged incubation with the microsomal enzyme, unlike the reaction catalysed by s-RNA nucleotidyltransferase, which stops when the specific terminal sequence has been synthesized.

Table 6 shows that the microsomal enzyme could attach CMP in positions on phosphodiesterase-treated s-RNA that normally accept the -pCpCpA sequence. As a result the incorporation of CMP in a subsequent incubation with s-RNA nucleotidyltransferase was decreased. Thus, when the total capacity of 5 μ g. of phosphodiesterase-treated

s-RNA with s-RNA nucleotidyltransferase was 95 μ moles of CMP, prior incorporation of 59 μ moles of CMP by the microsomal enzyme decreased this capacity by 45 μ moles. The control experiment showed that preincubation of the phosphodiesterase-treated s-RNA with the microsomal enzyme but without CTP did not affect the subsequent incorporation of [14 C]CMP by the s-RNA nucleotidyltransferase.

Location of CMP incorporated into s-RNA. Characterization of the reaction product in experiments similar to those shown in Table 6 confirmed that the microsomal enzyme attached CMP at acceptor sites for the -pCpCpA sequence (Table 7). Incubation of phosphodiesterase-treated s-RNA with s-RNA nucleotidyltransferase and [14 C]CTP gave a product from which [14 C]cytidine and 2'(3')-[14 C]CMP were recovered in equal amounts after alkaline hydrolysis (Table 7, Expt. 1a). When non-radioactive ATP was also present, only 2'(3')-[14 C]CMP (from internal CMP residues) was recovered (Table 7, Expt. 1b). These findings reflect the synthesis of s-RNA-pCpC and s-RNA-pCpCpA respectively from phosphodiesterase-treated s-RNA.

Incubation with the microsomal enzyme also synthesized terminal sequences consisting on average of about two CMP residues (Table 7, Expts. 2a and 3a). A subsequent incubation with s-RNA nucleotidyltransferase and excess of non-radioactive CTP (to dilute the incorporation of [14 C]CMP to a negligible level) decreased the amount of [14 C]cytidine recovered by about 60% (Table 7, Expt. 2b), indicating the attachment of non-radioactive CMP to about 60% of the terminal [14 C]CMP residues. The latter were presumably present in the form s-RNA-pC, because s-RNA nucleotidyltransferase under these conditions synthesizes only s-RNA-pCpC. Since essentially similar results were obtained when non-radioactive ATP was present during the second incubation (Table 7, Expt. 3b), the acceptor made by incubation with the microsomal enzyme was mainly s-RNA-pC and to a much smaller extent s-RNA-pCpC (which should accept AMP from s-RNA nucleotidyltransferase).

In other experiments attempts were made to demonstrate the attachment of CMP by the microsomal enzyme to -pCpC ends synthesized by s-RNA nucleotidyltransferase, by incubating first with the latter enzyme and then with the microsomal enzyme. These experiments were inconclusive because of the phosphodiesterase activity in the microsomal enzyme. On the other hand, it was shown that the microsomal enzyme did not attach CMP to s-RNA-pCpCpA. Thus s-RNA-pCpCpA labelled with [14 C]AMP in the terminal residue released 96% of its radioactivity as [14 C]adenosine

Table 7. *Location of CMP incorporated into s-RNA*

The incubations were as described in Table 6 with the following modifications: the first or the second incubation was omitted as shown, and non-radioactive CTP (700 μ moles/ml.) and ATP (60 μ moles/ml.) were added in the second incubation where indicated. Several replicate tubes were pooled to obtain sufficient radioactivity and the reaction product was characterized as described in Table 4. The recovery of radioactivity was essentially complete in all cases. The symbol pC* indicates a [14 C]CMP residue in the product.

Expt. no.	First incubation (microsomal enzyme)	Second incubation (s-RNA nucleotidyltransferase)	Radioactivity recovered (%)		Total radioactivity recovered (counts/min.)	Chain length	Product
			In cytidine	In CMP			
1a	Not incubated	Normal	49	51	934	2-0	s-RNA-pC*pC*
1b	Not incubated	Normal + ATP	< 1	100	990	—	s-RNA-pC*pC*pA
2a	Normal	Not incubated	49	51	573	2-0	s-RNA-pC*pC* (average)
2b	Normal	Normal + CTP	19	81	609	—	s-RNA-pC*pC+ s-RNA(pC*) _n
3a	Normal	Not incubated	58	42	1043	1-7	s-RNA-pC*pC* (average)
3b	Normal	Normal + CTP + ATP	20	80	1060	—	As in Expt. 2b + trace of s-RNA-pC*pC*pA

on alkaline hydrolysis, both before and after incubation under the standard conditions (Table 7) with the microsomal enzyme and non-radioactive CTP. This indicates that the single terminal pA residue did not act as a site of attachment for CMP residues.

DISCUSSION

In the presence of Mn^{2+} the microsomal enzyme attached all four ribonucleotides as short homopolymer chains to the 3'-end of the primer RNA, and therefore closely resembled the microsomal enzyme described by Wykes & Smellie (1966), except that the latter is Mg^{2+} -dependent. There is no evidence that either of these enzymes can make heteropolymers by replication of the RNA primer, unlike the virus-induced polymerase that is found in association with the microsomal fraction (e.g. Baltimore & Franklin, 1963). Like the enzyme of Wykes & Smellie (1966), other homopolymer-synthesizing enzymes from a variety of sources (see the introduction) are also mainly Mg^{2+} -dependent. A Mn^{2+} -dependent homopolymer synthesis by DNA-primed RNA nucleotidyltransferase (EC 2.7.7.6, 'RNA polymerase') is known to occur in reactions primed by DNA (Chamberlin & Berg, 1962, 1964; Burdon, 1963b; Fox & Weiss, 1964; Stevens, 1964) or by polyribonucleotides (Krakow & Ochoa, 1963; Fox, Robinson, Haselkorn & Weiss, 1964; Gomatos, Krug & Tamm, 1964; Niyogi & Stevens, 1965), but the reaction studied in the present paper was inhibited by denatured DNA and no DNA-primed synthesis of homo- or hetero-polymers could be demonstrated.

The incorporation of any one ribonucleotide by the microsomal enzyme was also inhibited by the other three ribonucleoside triphosphates, and

showed no primer specificity. In these respects the reactions were very similar to the Mn^{2+} -dependent incorporation of AMP and UMP by enzymes prepared from rat-liver supernatant (Klemperer, 1963a,b, 1965). Moreover, in both cases oligoribonucleotides were the best primers for AMP incorporation, and Mg^{2+} could replace Mn^{2+} for UMP incorporation. The fact that the supernatant enzymes for AMP and UMP incorporation were partially separable, and that the relative activities towards different ribonucleotides by the microsomal extract varied in different preparations and at different stages in the purification, suggests that separate enzymes for each ribonucleotide were present in the microsomal enzyme preparation.

CMP was incorporated by the microsomal enzyme into a variety of polyribonucleotides, and both native and phosphodiesterase-treated s-RNA were effective primers. Since s-RNA-pCpCpA could not itself act as an acceptor, other molecules present in native s-RNA must have primed the reaction. It is likely that these were partially degraded molecules. The presence of these is indicated by the fact that the native s-RNA had a capacity of 4 μ moles of AMP/mg. when incubated with s-RNA nucleotidyltransferase. Similarly degraded molecules would also be produced during the incubation with the microsomal enzyme because the latter was contaminated with phosphodiesterase.

[14 C]CMP residues were attached to phosphodiesterase-treated s-RNA in the position of the innermost residue of the normal -pCpCpA terminal sequence, since the product (s-RNA-pC) could itself accept CMP from s-RNA nucleotidyltransferase in a subsequent incubation (Table 7). The presence of a small proportion of s-RNA-pCpC capable of accepting AMP is also suggested by these findings.

The results in Table 7 show that s-RNA-pC, with a single [^{14}C]CMP residue capable of acting as an acceptor for CMP from s-RNA nucleotidyltransferase, represented about 60% of the [^{14}C]CMP residues incorporated in terminal positions by the microsomal enzyme, but only about 30% of the total [^{14}C]CMP incorporated by this enzyme. Since the overall chain length synthesized was two, much of the remaining 40% of the terminal [^{14}C]CMP was at the ends of newly synthesized chains of more than two [^{14}C]CMP residues. These chains may have been in the form of s-RNA-pCpCpC, i.e. attached in place of -pCpCpA, since the proportion of the [^{14}C]CMP in the form s-RNA-pC was insufficient to account for the inhibition of CMP incorporation by s-RNA nucleotidyltransferase observed in Table 6. Thus, if 30% of the incorporated [^{14}C]CMP was in the form s-RNA-pC and 70% in the form s-RNA-pCpCpC, then the inhibition of the subsequent CMP incorporation by s-RNA nucleotidyltransferase would have amounted to $30\% + (70 \times 2/3)\% = 77\%$ of the total incorporation by the microsomal enzyme, which corresponds closely to the value $(45/59 = 76\%)$ obtained from the results in Table 6.

It therefore appears that the microsomal enzyme could attach CMP in positions normally occupied by CMP and AMP in the -pCpCpA terminal sequence, but the new sequence synthesized in this way did not appear to contain a defined number of CMP residues. In contrast, s-RNA nucleotidyltransferase and an enzyme from thymus described by Hurwitz & Bresler (1961) both attach only -pCpC sequences, and do so only at specific sites on specific acceptor molecules. On the other hand, the enzyme from thymus nuclei described by Edmonds (1965) synthesizes terminal chains consisting of several CMP residues, but again appears to have a specific polynucleotide acceptor, unlike the microsomal enzyme, for which no specific primer has yet been found.

This work was supported in part by a Research Grant from the Medical Research Council and by a Research Studentship to M. G. P. from the Science Research Council.

REFERENCES

- August, J. T., Ortiz, P. J. & Hurwitz, J. (1962). *J. biol. Chem.* **237**, 3786.
- Baltimore, D. & Franklin, R. M. (1963). *J. biol. Chem.* **238**, 3395.
- Burdon, R. H. (1963a). *Biochim. biophys. Res. Commun.* **11**, 472.
- Burdon, R. H. (1963b). *Biochem. biophys. Res. Commun.* **13**, 37.
- Burdon, R. H. & Smellie, R. M. S. (1961). *Biochim. biophys. Acta*, **51**, 153.
- Canellakis, E. S. (1957). *Biochim. biophys. Acta*, **23**, 217.
- Canellakis, E. S., Gottesman, M. E. & Kammen, H. O. (1960). *Biochim. biophys. Acta*, **39**, 82.
- Canellakis, E. S. & Herbert, E. (1960). *Biochim. biophys. Acta*, **45**, 133.
- Chakravorty, A. K. & Biswas, B. B. (1965). *J. biol. Chem.* **240**, 4406.
- Chamberlin, M. & Berg, P. (1962). *Proc. nat. Acad. Sci., Wash.*, **48**, 81.
- Chamberlin, M. & Berg, P. (1964). *J. molec. Biol.* **8**, 708.
- Chung, C. W., Mahler, H. R. & Enrione, M. (1960). *J. biol. Chem.* **235**, 1449.
- Dalgarno, L., Martin, E. M., Liu, S. L. & Work, T. S. (1966). *J. molec. Biol.* **15**, 77.
- Edmonds, M. (1965). *J. biol. Chem.* **240**, 4621.
- Edmonds, M. & Abrams, R. (1960). *J. biol. Chem.* **235**, 1142.
- Edmonds, M. & Abrams, R. (1962). *J. biol. Chem.* **237**, 2636.
- Fox, C. F., Robinson, W. S., Haselkorn, R. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 186.
- Fox, C. F. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 175.
- Gomatos, P. J., Krug, R. M. & Tamm, I. (1964). *J. molec. Biol.* **9**, 193.
- Gottesman, M. E., Canellakis, Z. N. & Canellakis, E. S. (1962). *Biochim. biophys. Acta*, **61**, 34.
- Hecht, L. I., Zamecnik, P. C., Stephenson, M. L. & Scott, J. F. (1958). *J. biol. Chem.* **233**, 954.
- Herbert, E. & Canellakis, E. S. (1961). *Biochim. biophys. Acta*, **47**, 85.
- Hurwitz, J. & Bresler, A. E. (1961). *J. biol. Chem.* **236**, 542.
- Josse, J. & Kornberg, A. (1962). *J. biol. Chem.* **237**, 1968.
- Kammen, H. O., Klemperer, H. G. & Canellakis, E. S. (1961). *Biochim. biophys. Acta*, **57**, 175.
- Klemperer, H. G. (1963a). *Biochim. biophys. Acta*, **72**, 403.
- Klemperer, H. G. (1963b). *Biochim. biophys. Acta*, **72**, 416.
- Klemperer, H. G. (1965). *Biochim. biophys. Acta*, **95**, 251.
- Koerner, J. F. & Sinsheimer, R. L. (1957). *J. biol. Chem.* **228**, 1049.
- Krakow, J. S. & Ochoa, S. (1963). *Proc. nat. Acad. Sci., Wash.*, **49**, 88.
- Kuby, S. A., Noda, L. & Lardy, H. A. (1954). *J. biol. Chem.* **209**, 191.
- Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 447. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- McDonald, M. R. (1955). In *Methods in Enzymology*, vol. 2, p. 427. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Niyogi, S. K. & Stevens, A. (1965). *J. biol. Chem.* **240**, 2587.
- Rosset, R., Monier, R. & Julien, J. (1964). *Bull. Soc. Chim. biol., Paris*, **46**, 87.
- Smellie, R. M. S. (1965). *Biochem. J.* **94**, 2p.
- Stevens, A. (1964). *J. biol. Chem.* **239**, 204.
- Straus, D. B. & Goldwasser, E. (1961). *J. biol. Chem.* **236**, 849.
- Venkataraman, P. R. & Mahler, H. R. (1963). *J. biol. Chem.* **238**, 1058.
- Wykes, J. R. & Smellie, R. M. S. (1966). *Biochem. J.* **99**, 347.